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PRINCIPAL INVESTIGATOR: Kathy Helzlsouer, M.D.

CONTRACTING ORGANIZATION: Johns Hopkins University
Baltimore, Maryland 21205

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Kathy J. Helzlsouer, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Johns Hopkins University
Baltimore, Maryland 21205

E-Mail: khelzlso@jhsph.edu

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ABSTRACT

The purpose of this study was to enhance the resources of the existing population-based specimen bank by updating information, and expanding information collected in 1989. Particular emphasis was placed on updating risk factors for breast cancer so that gene-environment interactions leading to breast cancer may be investigated. Since the resources of serum banks become more valuable with time, as cohorts mature, providing more cases of cancer for investigation, it is important to ascertain storage stability over time. Therefore the second major objective of this proposal was to continue to obtain information on changes in the concentration of various analytes in plasma and blood cells associated with long-term storage at –70 C. The hypothesis tested was the null hypothesis that there will be no change in concentration with storage time.

From May through November 1989, a campaign (Clue II) was conducted in Washington County, Maryland, to collect blood for a specimen bank. A total of 32,898 persons participated. Participants donated 20 ml of blood, gave a brief medical history, completed a food frequency questionnaire and returned a toenail for trace metal studies. In order to use the specimen bank to its fullest potential, additional data was required, primarily on known and suspected risk factors for cancer. This information was obtained by three self-administered mailed questionnaires mailed approximately 18 months apart. The optical mark readable questionnaires included questions on smoking status, weight changes, use of exogenous hormones, medical conditions, detailed family history of cancer, screening history, history of breast biopsies, medical history, medication and vitamin use, exercise history and other potential risk factors for cancer particularly breast cancer. In an effort to expand the cancer registry population base to persons who reside outside of Washington County, participants who lived within a 30 mile radius were included in the follow-up study. This area includes all of Washington County and parts of surrounding counties, extending into Pennsylvania and West Virginia.

The response rates for each of the questionnaire mailings were 70 percent (1996) 64 percent (1998) and 64 percent (2000). Assays for micronutrients after 12 years of storage at –70 C are currently under way and a manuscript should be produced within a few months. As part of the hormone stability study, plasma that had been stored for 11 years was sent to the laboratory that had done the earlier assays and to another laboratory. Rank order correlation coefficients varied markedly by sex.

Keywords: breast cancer, storage effects, serum banks

FOREWORD

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N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Kathy J. Kilmer 03/26/01

PI - Signature Date

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INTRODUCTION

The purpose of this study was to enhance the resources of the existing population-based specimen bank by updating information, and expanding information collected in 1989. Particular emphasis was placed on updating risk factors for breast cancer so that gene-environment interactions leading to breast cancer may be investigated. The second major objective of this proposal was to continue to obtain information on changes in the concentration of various analytes in plasma and blood cells associated with long-term storage at –70° C.

BODY

Technical Objectives (Specific Aims)

To enhance the resources of the existing population-based specimen bank we:

1. Expanded information collected at Clue II in 1989.

2. Updated information collected at Clue II. For example, changes in marital status, smoking status, use of exogenous hormones, weight changes and dietary changes.

3. Obtained additional information relevant to breast cancer. For example, detailed cancer family history, number and timing of pregnancies, preventive health behavior and history of breast biopsies.

4. Expanded the cancer registry population base by including Clue II participants who reside outside of Washington County.

5. Continued the study of the effects of long-term freezer storage of plasma at 70° C on its content of antioxidant nutrients and hormones.

BACKGROUND OF PREVIOUS WORK

The Clue Specimen Banks

From August through November, 1974, a total of 25,802 serum specimens were collected in Washington County, MD for a research specimen bank (Clue I). Linkage of the records from this program to those of a private census in the summer of 1975 indicated that almost a third of the adult population of the county had participated. Participation was best in the age group 35 to 65 years, and was slightly better among females, the better educated, and non-smokers.

A second program was conducted from May through November, 1989 collecting 32,898 blood specimens (Clue II). Of these specimens, 8,395 were collected from individuals who had also participated in Clue I. Clue II participants donated 20 ml of blood, gave a brief medical history, completed a food frequency questionnaire and returned a toenail clipping for trace metal studies. The blood specimens have been stored at – 70° C. Somewhat greater participation was obtained among older persons, possibly because a free cholesterol test was offered as an incentive to participate.

In addition to storing two 5 ml aliquots of plasma, another 0.7 ml aliquot was preserved with 0.7 ml of 10 percent metaphosphoric acid to allow for subsequent ascorbic acid assays. The buffy coat (providing DNA) and an aliquot of red blood cells were also stored.

Clue I has been extensively used to examine the potential protective effect of specific micronutrients against the development of cancer including breast cancer (1). The role of endogenous hormones in the development of breast cancer has also been examined using the resources of this serum bank (2). With the maturation of the cohort from Clue II, the availability of DNA in addition to plasma, and the technological advances of molecular biology we plan to investigate etiologic, protective and susceptibility factors leading to the development of breast cancer. In order to use the specimen bank to its fullest potential we required additional information from participants on breast cancer risk factors such as family history of disease, age at pregnancy and parity change over time, history of benign breast disease, requiring institution of an active follow-up of the cohort. These risk factors should be taken into account in investigations of serologic precursors of susceptibility factors associated with breast cancer. The ability to identify and investigate families with multiple members affected by breast cancer is a valuable resource for studying the role and contribution of inherited susceptibility factors to the development of breast cancer. Since these inherited factors may be passed through the mother or father it is essential to obtain information on the family history of cancer of male and female participant in the cohort.

CANCER REGISTRY

A cancer registry for Washington County has been maintained since 1958, with records dating back to 1948. Its primary source is discharge records from the Washington County Hospital, the only general hospital in the county. Because of its well-equipped and staffed oncology service, the hospital tends to draw patients from surrounding counties rather than to lose them to other institutions. Cases are ascertained from death certificates of Washington County residents, which are under custody of our unit acting as a branch of the Health Department. Comparisons of observed cases in the populations that donated blood for the serum bank with the number expected on the basis of race-sex age specific rates from the SEER registries suggest that reporting is essentially complete for this sub-population. The only deficit is for stomach cancer; the only major excess is for prostate cancer. Records of reported county cancer cases are linked to the data- base containing serum bank donors.

METHODS

Eligible Population

Clue II participants living within a 30 mile radius of downtown Hagerstown were included in the active follow-up cohort. This geographic boundary included all of Washington County and parts of surrounding counties, extending into Pennsylvania and West Virginia. This boundary included 30,724 of the 32,989 Clue II participants. This boundary was chosen to form a homogenous cohort for long-term follow-up. Persons under the age of 13 at the time of participation and persons known to have died, were excluded from the follow-up cohort.

Follow-up Procedure

Clue II participants located in the geographic boundary of the long-term follow-up cohort were mailed a questionnaire in July 1996, March 1998, and May 2000. Addresses were updated prior to each newsletter and questionnaire mailing. Deceased Clue II participants were identified by linking the population to the State mortality data-base and by using obituaries from the local newspaper. Non-respondents were sent a post card reminder within 2 weeks of the initial questionnaire mailing followed by another questionnaire within 4 weeks of mailing. Telephone follow-up was carried out to clarify questionnaires completed by respondents and to administer the questionnaire to non-respondents. We found that telephone interviews were costly since it often required a number of calls to reach the participant at home.

In order to determine which types of follow-up procedures were most effective in increasing response prior to the first mailing in 1996 we conducted a controlled trial of the effect of length, incentives, and follow-up techniques on response to a self administered mailed questionnaire. Interventions tested included variations on length of the questionnaire, effect of a non-monetary incentive and effect of a postcard reminder versus a letter accompanied by a second questionnaire. Response rates were similar for short (16 questions, 4 pages) and long (77 questions, 16 pages) questionnaire groups. The non-monetary incentive did not improve response rates. The second mailing of a questionnaire was significantly better than a postcard reminder in improving response (23 percent versus 10 percent) (3). Attachment 1

REPORT ON STATUS OF TASK S-STATEMENT OF WORK

Task 1: Updating of addresses of Clue II participants.
Tracing of Participants

Participants of the 1989 Clue II cohort who met the study criteria for inclusion in the long-term follow-up, were identified by determining geographic boundaries. A manual search of phone books, the Polk City Directory for Hagerstown and the Hill-Donnelly Cross Reference Directory for Washington County was conducted prior to each questionnaire mailing.

A newsletter was mailed to each participant approximately one month prior to the questionnaire mailing with an address correction requested from the postal service. (Attachments 2 and 3) This method proved effective as a cost saving measure, in that it cost less to mail the newsletter to obtain an up-to-date address from the postal service than it would have cost to use the questionnaire for this purpose. Names changes for females were updated by searching archived published wedding announcements when feasible. For questionnaire mailings subsequent to 1996 the contact information provided by respondents to the questionnaire was followed up on to obtain a current address.

Vital status was determined by linking the listing of participants to the mortality data obtained from the State Health Department as well as from death certificates housed in our unit as part of the Research Unit of the Health Department. 2,697 participants who were residents of Washington County are known to have died during the follow-up period 1996-2000. 157 out-of-county residents are known to have died, however this data is less complete in that we do not obtain mortality data for persons residing outside of the county. 140 participants were identified as being deceased when a search of the Social Security Death Index was conducted.

A listing was sent to the Maryland Motor Vehicle Administration in order to update addresses of Maryland residents. An updated address was obtained for approximately 30 percent of the records sent to the MVA. For the Odyssey Cohort (persons who participated in Clue I and Clue II) a credit agency search was implemented (CBA Information Services) which resulted in identifying updated addresses for 117 participants. 15 percent of participants (4,480) in the follow-up cohort continue to be lost to follow-up. Table 1 shows the number (and percent) of persons who were not located by age, gender, education and geographic location (1996). The highest percentage of persons lost to follow-up were persons under age 25 years.

Task 2: Designed and coordinated the printing of and three questionnaires.

The Serologic Precursors Committee which is composed of study investigators met numerous times to design each of the three questionnaires. The first questionnaire, which was the longest in length of the three, was a 76-item optical mark readable questionnaire which was printed by Scantron . (Attachment 4) The second questionnaire, a 31-item, optical mark questionnaire was printed by National Computer Systems. (Attachment 5) The third and final questionnaire was a 46-item questionnaire, also printed by National Computer Systems. (Attachment 6)

Task 3: Mailed questionnaires to study participants

Each of the questionnaires were pilot tested on participants who resided outside of the 30 mile radius to assess the clarity of questions and the readability of the questionnaire. The completed questionnaires were coded and entered into a computer data base to evaluate the effectiveness of methods for coding and data entry. Changes in the questionnaire design were made as a result of the pilot testing. The first questionnaire was mailed in July 1996, the second in March 1998 and the final in May 2000. Age and education were associated with response rate, with the highest response being among persons in the 65-74 age group. Response was higher among females and married persons. Overall, response was higher among persons residing within Washington County compared to those residing outside of the county. (Table 2) Overall response rates were 69.9 percent in 1996, 64.3 percent in 1998 and 63.6 percent in 2000. Fifty-nine percent of the respondents responded to all three questionnaire mailings. (Table 3) Non-respondents were sent a post card reminder within 2 weeks of the initial questionnaire mailing, followed by another questionnaire within 4 weeks of mailing.

Task 4: Editing and scanning of optical marked machine- readable questionnaires.

Editing and scanning has been completed for the first two questionnaire mailings and is 75 percent complete for the third questionnaire mailing. Tabulations and code books have been completed for the first two questionnaire mailings. Tables 4 through 15 contain tabulations related to changes in demographic status, conditions reported by respondents, and specific risk factors related to breast cancer. This data has been used for numerous publications and presentations, which are listed later in this report. Some of the student projects that are currently in progress and using this data are:

Serum antibodies to polyomavirus and incident primary brain cancer.
Insulin resistance in the etiology of colorectal cancer.
Vasectomy as a risk factor for prostate cancer.
Screening prevalence for colon cancer.
Organochloride exposure and breast cancer.
Prostate cancer and calcium intake.
Trends in physical activity in Washington County.
Blood pressure, incident myocardial infarction and mortality in Washington County.
Passive smoke exposure and breast cancer.

Task 5: Linkage of follow-up data to other data bases. Verification of vital status.

The cancer registry population base was expanded to include participants who reside outside of Washington County. (Table 14) Mortality data was linked to the Clue II follow-up cohort, to identify 2,697 persons who had died.

Task 6: Telephone interviews to non-respondents.

In July of 1997, after data entry and scanning of questionnaires was completed, we began a telephone follow-up of non respondents. The follow-up group was limited to Washington County residents at baseline (Clue II, 1989) who currently resided in the county, and for whom we had a telephone number listed in our data base. We established this inclusion criteria so that we could contact as many persons as possible in a limited time period, and reduce costs by eliminating the need for long distance calls. Lists of non-respondents were printed which included the name, address, date of birth and telephone number of the participant. Lists were distributed to staff according to alpha-numeric participant number. Staff were instructed to make one attempt to contact the participant. Contact was made with 2,135 persons. Of that group a shortened version of the questionnaire was administered over the telephone to 759 persons. I was estimated that it cost approximately $5.00 in staff time for each interview.

Task 7: Study of effects of stability of plasma (and serum) components when stored at -70° C.

Participants in Clue II donated 20 ml of blood in 1989. This was collected in a 20 ml vacutainer containing heparin and was stored at 4° C until it was delivered to the laboratory within a few hours after being drawn. After centrifugation, 0.7 ml of plasma was added to 0.7 ml of 10% metaphosphoric acid for ascorbic acid assays. The remaining plasma was divided into two equal aliquots, the buffy coat was removed, and 2 ml of red blood cells was saved. Each of these specimens was placed in 5 ml cryotubes and promptly frozen at -70° C.

For the stability studies of micronutrients and hormones, pools of plasma were created from specimens donated by persons who lived far outside the study area and who had participated near the end of the campaign. For ascorbic acid assays, 40 pools were created, each containing the plasma from four individuals. For the other assays, 16 pools were created, again each containing the plasma from four persons. For all of the analytes, one quarter of the pools was composed of plasma from each of the four sex-age groups (males born 1910-1939; males born 1949-1969; females born 1910-1939; and females born 1940-1969). A large quality control pool was also created so that four quality control aliquots could be included in the ascorbic acid assays, and two in the other assays.

Since the previous report, three papers related to storage effects have been published. A study of temperature variations within chest-type mechanical freezers has been published (4). For 27-cubic foot freezers, the temperature differential was 15.9° C between top and bottom layers. For 7-cubic foot freezers, the differential was 10.1° C. Such differences in temperature could be important when specimens are stored for very long periods of time.

The effects of repeated freeze-thaw cycles were investigated for micronutrients (retinol, major carotenoids, ascorbic acid, and tocopherols), and hormones (androstenedione, estrone, estradiol, FSH, progesterone, testosterone, six hormone binding globulin, DHEA and DHEA-S). Assays were done after 1, 2, 3, 4, 6, and 10 freeze-thaw cycles. Freezing was to -70° C and thawing to room temperature. The mean change per freeze-thaw cycle and its accompanying exposure to room temperature for 30 minutes was less than 4 percent for all analytes, and less than 2 percent for all except five (estrone in plasma and serum; estradiol in serum; and sex hormone binding globulin and DHEA-S in plasma). Changes tended to be least for the first three freeze-thaw cycles. This paper has been published (5).

A study of the repeatability of micronutrient concentrations in serum and plasma has also been published (6). Following is the abstract of that paper.

"Community-wide programs to collect blood for a research serum bank were carried out in Washington County, Maryland in 1974 and 1989. Of the 8,395 persons who participated in both programs, 64 were controls in a nested case-control study of the association of antioxidant micronutrients with subsequent breast cancer, and 30 and 166 were controls in similar studies of lung and prostate cancer. Assay results for five carotenoids, two retinoids, and two tocopherols in samples of blood collected 15 years apart were thus available for comparisons of micronutrient concentrations. The mean Spearman rank order correlation coefficient for all comparisons was 0.44, with two coefficients greater than 0.60 and two less than 0.30. Blood pressure readings at the two blood collections had a mean rank order correlation coefficient of 0.46. Because blood pressure readings in 1974 were shown to be significantly predictive of atherosclerosis 15-18 years later, the present results suggest that ranked concentrations of antioxidant micronutrients from a single sample are sufficiently representative to be used as predictors of subsequent concentrations and are thus suitable for assessment as risk factors for subsequent illnesses."

A manuscript for the fourth study is currently in preparation. This deals with the stability of the steroid hormones estrone, estradiol, progesterone, and testosterone, and sex hormone binding globulin in plasma stored for 11 years at -70° C. The mean percentage change per year (based on the calculated midpoint value at 65.5 months) was 0.10 for estrone; -0.14 for estradiol; 0.90 for progesterone; 0.27 for testosterone; and -0.11 for sex hormone binding globulin. These findings suggest that long term storage of plasma has sufficiently little effect on steroid hormone concentrations that it can probably be ignored, especially since it will affect the cases and control similarly.

As part of the hormone stability study, plasma that had been stored for 11 years was sent to the laboratory that had done the earlier assays and to another laboratory. Correlation coefficients were 0.70 for estrone; 0.69 for estradiol; 0.986 for testosterone; and 0.59 for sex hormone binding globulin. However, rank order correlation coefficients varied markedly by sex as is shown in the following table

	Spearman rank order correlation coefficients			
Sex	**Estrone**	**Estradiol**	**Testosterone**	**Sex hormone binding globulin**
Male	-0.13	-0.34	0.90	-0.17
Female	0.80	0.80	-0.02	0.76

We are still uncertain about the meaning of these sex-related differences.

Assays for micronutrients after 12 years of storage at -70° C are currently under way and a manuscript should be produced within a few months.

It had been originally planned to do a similar study on lipoproteins. Unfortunately, the participating laboratory changed its methods after a few years had passed, and the results of the two methods showed such poor correlation that this part of the study was abandoned.

KEY RESEARCH ACCOMPLISHMENTS/REPORTABLE OUTCOMES

The key accomplishments of this project are the echancement of the resources of the existing data base by expanding and updating the information collected at baseline. This data has been used in the implementation of numerous studies which have resulted in publication. See listing of publications, which are part of this report. We have also made data available to researchers external to the organization. Examples are as follows:

EXTERNAL COLLABORATORS

Food and Drug Administration, Micronutrients and endometrial cancer.
National Cancer Institute, Insulin Growth Factor and Prostate Cancer.
Oxford, Breast Cancer Hormones.
3M St. Paul Minnesota, Effects of fluorocarbons on lipid metabolism in the Clue population.
University of Minnesota, Risk factors for breast cancer in the Atherosclerosis Risk In Communities (ARIC) study.
University of Washington, Risk factors for cancer in an established cohort of the elderly in the Cardiovascular Health Study (CHS).
Fred Hutchinson Cancer Research Center, Epstein barr virus antibody titers and breast cancer risk.
Tim O'Brien ODC prostate cancer

KEY PERSONNEL

Kathy J. Helzlsouer
Han-Yao Huang
Reiko Sata
Gloria Zepp
Sandra Hoffman
Judith Hoffman-Bolton
Clara Krumpe
Kitty Spoonire
Joan Vafakas
Ann Conrad
Kathie Niswander
Alyce Burke
Jane Hixson

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Alberg AJ, Landrigan J, May JW, Hoffman SC, Gitchell J, Comstock GW, Helzlsouer KJ. Nicotene replacement therapy use among a cohort of smokers. Submitted

Sato R, Fried LP. Helzlsouer KJ, Hoffman SC, Norkus EP, Comstock GW. A cross-sectional study of vitamin C and cognitive function in older adults: The differential effects of gender. Submitted

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ABSTRACTS

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CONCLUSIONS

Tables 4 through 13 contain tabulations related to changes in demographic status, conditions reported by respondents, and specific risk factors related to breast cancer. The cancer registry population base was expanded to include participants who reside outside of Washington County, (Table 14) and a count of cancer cases who meet the usual criteria for inclusion in serologic studies was made for some of the most common cancers. (Table 15) These data are available as a resource to investigate gene-environment interactions leading to breast cancer. Storage stability over time has been ascertained. Assays after approximately 4 years of storage show that there was no indication of any meaningful losses of ascorbic acid, retinol, alpha-tocopherol, or gamma-tocopherol.

Table 1 Active follow-up of Clue II participants

Number (and percent) **unable** to locate by age, sex, education and geographic location of total sample in **first** questionnaire mailing

	Number in sample	Unable to locate rate %
Total	4796	17.0
Age (years)		
<25	3809	81.7
25-34	257	10.9
35-44	284	6.9
45-54	207	3.9
55-64	116	2.6
64-74	75	1.7
75+	48	1.7
Sex		
Male	1998	17.0
Female	2798	17.1
Education (years)		
<8	360	15.8
9-11	752	19.2
12	1966	15.7
13+	1712	18.2
Missing	6	---
Geographic location		
Washington County	3560	15.7
Outside of Washington County	1236	22.6

Table 2 Active Follow Up Response Rates
By baseline (1989) characteristics of responders by year of follow-up

		1996		1998		2000	
Number surveyed		23374		22605		21962	
Overall response rate		69.89		64.28		63.56	
		%	N	%	N	%	N
Gender	Male	65.72	6418	61.70	5800	60.55	5509
	Female	72.89	9919	66.12	8730	65.68	8449
Residence	WC	72.71	13914	66.06	12195	65.06	18093
	Other	57.17	2423	56.33	2335	56.50	3869
Age (Years)	<35	56.13	3197	47.79	2673	48.69	2718
	35-44	66.12	3200	60.55	2889	60.27	2897
	45-54	74.47	3501	69.88	3218	70.41	3244
	55-64	80.97	3528	78.19	3292	76.36	3088
	65-74	78.55	2501	73.88	2161	70.46	1810
	75 +	69.02	410	59.52	297	57.26	201
Race	White	70.29	16194	64.65	14407	63.89	13835
	Black	41.15	100	36.05	84	35.06	81
	Other	47.06	40	43.37	36	52.70	39
Marital Status Males	Married	70.11	5318	66.49	4861	65.23	4624
	Not Married	50.25	1085	44.61	923	43.74	870
Females	Married	75.82	7132	69.07	6353	68.25	6179
	Not Married	66.31	2769	59.30	2362	59.46	2251
Education Males	<12 Years	57.79	1183	51.23	979	50.51	893
	12 Years	64.80	2748	60.78	2483	59.86	2407
	> 12 Years	71.49	2478	68.67	2330	66.77	2202
Females	<12 Years	64.39	1962	56.41	1628	55.48	1508
	12 Years	73.26	4645	65.62	4058	65.73	4017
	> 12 Years	78.47	3302	73.62	3034	72.46	2915

Table 3 Participation by questionnaire mailing

Number and per cent of those participating at least once (18760)

	N	%
1996, 1996, 2000	11135	59.3
1996 only	2091	11.1
1998 only	667	3.6
2000 only	1073	5.7
1996, 1998	2044	10.9
1998, 2000	683	3.6
1996, 2000	1067	5.7

Table 4 Self Reported Medical Conditions Active Follow-up 1996-1998
Missing counted as negative response

	1996		1998	
Number of responders	16337		14529	
Number Men	6418		5799	
Number Women	9919		8730	
	Number	%	Number	%
Medical Conditions				
Diabetes diagnosis	1264	7.74	1197	8.24
High cholesterol diagnosis	5682	34.78		
Heart attack diagnosis	982	6.01	875	6.02
Angina pectoris diagnosis	805	4.93	602	4.14
Stroke diagnosis	361	2.21	372	2.56
Transient ischemic attack diagnosis	256	1.57		
Peripheral artery disease diagnosis	417	2.55	307	2.11
Osteoporosis diagnosis	848	5.19	814	5.60
Hip fracture diagnosis	208	1.27	166	1.14
Wrist or Colles' fracture diagnosis	891	5.45	759	5.22
Fibrocystic disease Women	2650	26.72	1957	22.42
Endometriosis diagnosis Women	875	8.82	695	7.96
Uterine fibroid diagnosis Women	1247	12.57	911	10.44
High blood pressure diagnosis	4878	29.86		
Migraine headache diagnosis	2181	13.35		
Thyroid Disease	1221	7.47		
Rheumatoid arthritis diagnosis	1233	7.55		
Colon Polyp	1112	6.81	1336	9.2
Gallbladder disease	1832	11.21		
Gastric or duodenal ulcer diagnosis	1280	7.83		
Macular degeneration of the retina	321	1.96	273	1.88
Cataract diagnosis	2091	12.80	1967	13.54
Asthma diagnosis	1263	7.73		
Emphysema or chronic bronchitis	1023	6.26		

Medical Conditions	Number	%	Number	%
Diverticulitis diagnosis	974	5.96		
Parkinson's disease diagnosis	70	0.43		
Kidney stone diagnosis	1319	8.07		
Ulcerative colitis/Crohn's disease	218	1.33		
Self reported cancer				
Bladder cancer			57	0.39
Breast cancer diagnosis Women	325	3.28	345	3.95
Cervical cancer diagnosis Women	173	1.74	119	1.36
Cancer of uterus Women	177	1.78	111	1.27
Ovary cancer Women	64	0.65	59	0.68
Leukemia			17	0.12
Colon cancer diagnosis	169	1.03	163	1.12
Lung cancer diagnosis	52	0.32	45	0.31
Lymphoma	28	0.17	53	0.36
Melanoma diagnosis	267	1.63	144	0.99
Skin - basal or squamous			712	4.90
Basal	895	5.48		
Squamous	230	1.41		
Pancreas cancer			11	0.08
Prostate cancer diagnosis Men	223	3.47	235	4.35

Table 5

Changes in marital status from Clue II (1989) to the active follow-up

	Status reported in the active follow-up				
Baseline Status	**Nev**	**Mar**	**Wid**	**Div**	**Sep**
never married	66.3	29.7	0.2	2.7	1.1
married	0.1	90.6	5.3	2.6	1.3
Other	1.6	13.7	48.0	34.8	1.9

Table 6 Percent currently smoking cigarettes, by race, sex, and age group at baseline (1989)

Age	Black females		Black males		White females		White males	
	N	% smoking	N	% smoking	N	% smoking	N	% smoking
18-24	16	18.8	9	22.2	976	27.6	623	27.5
25-34	36	33.3	33	36.4	2141	25.8	1504	27.7
35-44	44	45.5	43	37.2	2628	20.5	1902	25.5
45-54	31	29.0	26	42.3	2395	22.0	1760	21.0
55-64	21	23.8	17	47.3	2391	17.2	1684	14.9
65-74	13	7.7	8	25.0	1883	10.7	1396	10.0
75+	3	0.0	2	0.0	770	4.3	436	5.3
All	164	31.1	138	37.0	13184	19.2	9305	19.9

Table 7 Comparison of current smoking in 1989 and 1998 among white participants in both surveys by age group

Age 1989	White females			White males		
	N Age Group	1989 % smoking	1998 % smoking	N Age group	1989 % smoking	1998 % smoking
18-24	309	17.48	14.89	157	16.56	16.56
25-34	903	16.83	14.73	530	19.25	13.23
35-44	1409	15.61	11.98	879	19.68	15.60
45-54	1543	17.50	12.05	1050	18.48	13.04
55-64	1651	13.75	7.93	1123	11.31	6.86
65-74	1089	8.36	4.59	743	6.06	3.36
75+	233	3.86	1.72	137	5.11	2.92
All	7140	14.3	10.1	4619	14.6	10.3

Table 8 Comparison of overweight BMI among participants at baseline in 1989 and 1996 survey

Age in 1989	Males			Females		
	N age group	BMI 1989 % >27.8	BMI 1996 % >27.8	N age group	BMI 1989% >27.3	BMI 1996 % >27.3
18-24	194	16.5	37.1	373	14.8	30.6
25-34	592	26.0	36.5	1046	25.5	37.5
35-44	951	36.0	49.2	1564	31.5	44.4
45-54	1110	39.5	46.1	1658	37.3	48.6
55-64	1182	34.3	38.6	1728	37.6	43.6
65-74	835	27.9	27.0	1187	34.8	35.1
75+	159	17.0	16.4	295	23.7	19.7
All	5023	32.7	41.2	7851	32.5	39.3

Table 9 Exercise over whole life by sex and age as reported in 1998 follow-up questionnaire (% years over whole life)

	Males				Females			
	<44	**45-64**	**65-74**	**75+**	**<44**	**45-64**	**65-74**	**75+**
<1	5.9	7.9	10.2	11.4	12.2	15.1	18.7	26.7
1-5 years	8.2	11.7	9.9	11.4	20.0	17.1	12.9	13.9
6-10 years	13.9	10.0	6.4	7.9	17.5	12.2	7.4	8.0
11-15 years	14.4	8.9	4.8	4.6	14.4	9.3	7.8	4.3
16-20 years	18.3	12.4	9.4	5.9	16.7	12.3	9.3	8.2
20+ years	39.4	49.2	59.4	58.8	19.2	34.0	43.9	38.9
N per age Group	734	1851	1023	648	1311	2841	1366	814

Table 10

Mean daily servings of fruits and vegetables by gender, age, smoking status, marital status and education.

	Orange juice	Grapefruit juice	Other juice	Fruit	Salad	Fried potatoes	Baked potatoes	Other vegetables	Total per day (not fried pot)	% >=5 servings a day
Gender										
Men	.41	.08	.17	.75	.39	.18	.32	.90	3.02	14.6
Women	.40	.07	.18	.95	.46	.11	.33	1.09	3.48	21.9
Age										
Men										
<35	.40	.04	.22	.51	.30	.29	.23	.76	2.46	8.5
35-44	.33	.04	.18	.63	.30	.23	.28	.90	2.65	11.6
45-54	.36	.07	.15	.64	.37	.21	.30	.86	2.75	11.2
55-64	.40	.10	.15	.80	.43	.15	.32	.92	3.12	16.1
65-74	.47	.09	.19	.89	.44	.13	.34	.96	3.39	18.2
75+	.51	.09	.20	.91	.45	.11	.39	.88	3.42	18.6
Women										
<35	.35	.04	.25	.68	.37	.20	.26	.91	2.86	14.0
35-44	.27	.05	.16	.76	.39	.15	.29	1.07	3.00	16.9
45-54	.35	.06	.16	.90	.46	.12	.32	1.10	3.35	19.6
55-64	.43	.08	.17	1.03	.49	.10	.34	1.14	3.67	23.7
65-74	.46	.08	.17	1.16	.51	.08	.38	1.10	3.87	27.0
75+	.56	.09	.22	1.10	.49	.06	.40	1.08	3.94	28.7
Smoking										
Men										
Never	.44	.07	.19	.80	.40	.19	.32	.94	3.16	16.4
Former	.42	.09	.17	.79	.42	.15	.33	.91	3.13	15.5
Current	.29	.06	.13	.49	.30	.19	.29	.73	2.29	6.4
Women										
Never	.41	.07	.18	1.00	.46	.11	.34	1.12	3.57	23.0
Former	.41	.07	.17	1.03	.51	.10	.34	1.12	3.66	24.7
Current	.30	.07	.16	.65	.40	.14	.31	.89	2.78	12.7
Marital Status										
Men										
Married	.41	.08	.17	.78	.40	.17	.33	.92	3.08	15.4
Not	.42	.06	.19	.61	.34	.22	.28	.81	2.70	10.6
Women										
Married	.40	.07	.17	.98	.48	.11	.35	1.11	3.55	22.7
Not	.39	.07	.20	.89	.42	.12	.30	1.01	3.28	19.9
Education										
Men										
<HS	.39	.06	.19	.74	.32	.18	.34	.78	2.83	12.4
HS	.37	.07	.15	.71	.36	.18	.32	.84	2.82	11.8
>HS	.45	.08	.19	.81	.46	.17	.31	1.00	3.30	18.3
Women										
<HS	.41	.07	.20	.84	.38	.13	.34	.93	3.16	17.8
HS	.38	.07	.16	.89	.42	.12	.34	1.00	3.25	18.0
>HS	.41	.08	.20	1.09	.55	.10	.33	1.26	3.90	28.6

Odds ratio for consuming 5+ fruits/vegetables a day

Gender	OR	CI
Males	1.00	
Females	1.64	1.49-1.81

	Males N=4839		Females N=7133	
Age	**Crude OR**	**Adjusted**	**Crude OR**	**Adjusted**
<35	1.00	1.00	1.00	1.00
35-44	1.41	1.24	1.25	1.09
45-54	1.37	1.21	1.50 *	1.32
55-64	2.08 *	2.00 *	1.91 *	1.88 *
65-74	2.40 *	2.30 *	2.27 *	2.29 *
75+	2.47 *	2.28 *	2.47 *	2.43 *
Smoking				
Never	1.00	1.00	1.00	1.00
Former	0.94	0.82	1.09	1.00
Current	0.35 *	0.38 *	0.49 *	0.52 *
Marital status				
Married	1.00	1.00	1.00	1.00
Not married	0.65 *	0.85	0.84 *	0.91
Education				
<HS	1.00	1.00	1.00	1.00
HS	0.94	1.06	1.01	1.07
HS+	1.58 *	1.79 *	1.85 *	2.06 *

* P <.01

Table 12 Prevalence of breast cancer risk factors reported by female respondents to Clue II 1996 follow-up questionnaire

Risk Factor	Number	%
Age of first Menarche		
<=10	522	5.3
11	1293	13.0
12	2712	27.3
13	2784	28.1
14	1263	12.7
15	610	6.1
16	444	4.5
17	97	1.0
18+	32	0.3
Not stated	162	1.6
Menopausal Status		
Premenopausal	3022	30.5
Natural menopause	3665	36.9
Surgical menopause	2763	27.9
Other/ Not stated	469	4.7
Ever use birth control pills		
Yes	4621	46.6
No	5103	51.4
Not stated	195	2.0
Ever Take Estrogens		
Yes	2845	28.7
No	6782	68.4
Not stated	292	2.9
Ever Take Progestins		
Yes	1189	12.0
No	8232	83.0
Not stated	498	5.0
Ever been pregnant		
Yes	8341	84.1
No	1480	14.9
Not stated	98	1.0

Risk Factor	Number	%
Did you breast feed		
Yes	3601	44.0
No	4584	56.0
How many months breastfeeding		
<6 months	1243	34.5
6-12 months	1147	31.9
13 months - 24 months	645	17.9
25 months - 36 months	262	7.3
more than 36 months	195	5.4
Not stated	109	3.0
Age at first birth		
<18	725	8.9
18-19	1436	17.7
20-24	3591	44.2
25-29	1737	21.4
30-34	497	6.1
35+	133	1.6
Number of births		
1	1792	22.2
2	3078	38.1
3	1753	21.7
4	831	10.3
5	317	3.9
6+	311	3.8
Had a breast biopsy		
Yes	1683	17.0
No	7790	78.5
Not stated	446	4.5
Had a mammogram (2001 data)		
Yes	4666	77.0
No	1229	20.3
Not stated	165	2.7

Table 13 Family History of Breast Cancer Reported in 1996

Diagnosed with breast cancer in Cancer Registry	No. with family history	Percent of women responding to 1996 questionnaire
No. responding to questionnaire	201	
Mother's mother	9	4.5
Mother	18	9.0
Sister	20	10.0
Daughter	4	2.0

Table 14

Non Resident at Clue II-CA Diagnosis on Round 2 1998

Total	259
Site	
Bladder	5
Breast	42
Cervix	11
Colon	20
Leukemia	1
Lung	7
Lymphoma	4
Melanoma	21
Ovarian	7
Prostate	37
Skin	89
Uterine	13
Other CA	23

38 Dx WCH
195 Dx not WCH

26 Blank

16 / 259 Clue II DCQ = CA

Table 15 Cancer Registry* who meet usual criteria for inclusion in study

Cancer site	No. of cases	Response to follow-up questionnaire		
		1996	1998	2000
Breast	274	201	188	159
Colon/Rectum	173	105	82	70
Lung	186	84	51	22
Prostate	284	207	186	166
Endometrium	62	42	36	35
Ovarian	47	28	25	18

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A BRIEF ORIGINAL CONTRIBUTION

Controlled Trial of the Effect of Length, Incentives, and Follow-up Techniques on Response to a Mailed Questionnaire

Sandra C. Hoffman, Alyce E. Burke, Kathy J. Helzlsouer, and George W. Comstock

Mailed questionnaires are an economical method of data collection for epidemiologic studies, but response tends to be lower than for telephone or personal interviews. As part of a follow-up study of volunteers who provided a brief health history and blood sample for a blood specimen bank in 1989, the authors conducted a controlled trial of the effect of length, incentives, and follow-up techniques on response to a mailed questionnaire. Interventions tested included variations on length of the questionnaire, effect of a non-monetary incentive, and effect of a postcard reminder versus a letter accompanied by a second questionnaire. Response was similar for the short (16-item, 4-page) and long (76-item, 16-page) questionnaire groups. The non-monetary incentive did not improve the frequency of response. The second mailing of a questionnaire was significantly better than a postcard reminder in improving responses (23% vs. 10%). It is important to systematically test marketing principles to determine which techniques are effective in increasing response to mailed questionnaires for epidemiologic studies. *Am J Epidemiol* 1998;148:1007–11.

data collection; epidemiologic methods; health surveys; incentives; motivation

Self-administered, mailed questionnaires are a major source of data for epidemiologic studies. Compared with telephone and personal interviews, mailed questionnaires are more time- and cost-efficient to administer, and are particularly well suited for large or geographically dispersed study populations (1). Response to sensitive questions tends to be lower in mailed questionnaires than in telephone or in-person interviews, and bias is decreased when a questionnaire is mailed because the interviewer does not influence the response (1, 2).

The major disadvantage of mailed health-related questionnaires compared with telephone or in-person interviews is that there is limited opportunity for study staff to establish rapport with the potential respondent, and thereby influence motivation (2, 3). In order to maximize response, epidemiologists often use more expensive data collection techniques such as telephone and personal interviews (4).

The implementation of effective techniques that have been shown to increase response to mailed questionnaires would enhance the usefulness of this more cost-effective method of data collection for epidemiologic research. Surprisingly, epidemiologists have done comparatively little to study the practical aspects of the design and mailing of self-administered mailed questionnaires. Most studies have been conducted by market researchers and reported in journals that epidemiologists are unlikely to read, such as public relations and marketing journals.

Market research studies conducted over the past 20 years, along with a handful of epidemiologic studies, have evaluated the effectiveness of various techniques in increasing response to mailed questionnaires. The most frequently examined techniques are type and use of a cover letter for prenotification, use of incentives, type and frequency of respondent contact when following up on nonrespondents, type of postage, sponsoring agency, and questionnaire characteristics. The results of a meta-analysis of 82 articles (5) indicates that response rates can be increased by prenotification and follow-up, university sponsorship, a small cash incentive, first class outgoing postage, stamped return postage, and questionnaire color. Research results show a wide variability in the effects of the various incentives and prompts, which suggests that the generalizability of these interventions from one population and one questionnaire to the next is limited (3, 4)

As part of a follow-up study of volunteers who provided a brief health history and blood sample for a

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From the Department of Epidemiology, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, MD

Reprint requests to Sandra C. Hoffman, Training Center for Public Health Research, P.O. Box 2067, Hagerstown, MD 21742–2067.

specimen bank in 1989, we conducted controlled trials of three procedures to increase response to a questionnaire. The three investigations involved tests of the effect on response of questionnaire length, nonmonetary incentives, and the inclusion of a second questionnaire in a reminder notice. These trials were conducted as part of the pilot test of the proposed follow-up questionnaire.

MATERIALS AND METHODS

From May through November 1989, a campaign (CLUE II) was conducted in Washington County, Maryland, to collect blood for a specimen bank. A total of 32,898 persons participated. CLUE II participants donated 20 ml of blood, gave a brief medical history, completed a food frequency questionnaire, and returned a toenail clipping for trace metal studies. In return, all participants received a free serum cholesterol test. In order to use the specimen bank to its fullest potential, we required additional data, primarily known and suspected risk factors for cancer. This information was to be obtained by a self-administered mailed questionnaire. The 76-item optical mark readable questionnaire took approximately 20 minutes to complete. It included questions on family history of cancer in first- and second-degree relatives, reproductive history, screening history, medication and vitamin use, and history of medical conditions and surgery.

CLUE II participants who lived within a 30 mi (48.3 km) radius of the junction of Interstate highways I-70 and I-81 were the active follow-up cohort. This area includes all of Washington County and parts of surrounding counties, extending into Pennsylvania and West Virginia. This enclosed area includes 30,724 residents of the 32,898 CLUE II participants and was chosen to form a cohort for the long-term follow-up. Persons under age 18 years at the time of follow-up and persons known to have died were excluded, leaving 28,411 persons in the follow-up cohort.

The questionnaire was pilot tested on the 2,174 CLUE II participants who resided outside of the 30 mi (48.3 km) radius. In addition to assessing the clarity of questions and readability of the questionnaire, the pilot test was designed to test the effects of questionnaire length on response. Seventy percent of the pilot group (n = 1,504) were sent the full questionnaire consisting of 76 items on 16 pages. Thirty percent (n = 648) were sent a shorter version of the questionnaire with only the first four pages of the full questionnaire with 16 items on medical history and family history of cancer. Participants were assigned to a questionnaire group based on the terminal digit of their study number. The groups were similar in age, sex, and years of education. Questionnaires were machine-metered and mailed first-class during a 5-day period. Postage-paid self-addressed envelopes for return of the questionnaires were enclosed. Returned questionnaires with address corrections were promptly mailed to the new address. The questionnaires returned as non-deliverable (n = 671) were excluded from the analysis. The percent of non-deliverable questionnaires was high (30 percent) because the questionnaires were mailed to the address obtained 6 years earlier at the time of participation. Addresses had not been updated for this group prior to mailing because they were not part of the follow-up cohort. The cutoff period for recording responses was 21 days after the initial mailing.

Also prior to the mass mailing of the questionnaire to the entire follow-up cohort, a second pilot study was conducted to test the effect of including a newspaper article, and/or a special pencil on the initial response to the questionnaire. The entire study group (n = 28,411) was divided into five geographic regions by zip code. Within Washington County the zip codes represented persons who resided within the city of Hagerstown and surrounding towns. The study sample also included persons whose zip codes were outside of Washington County but within a 30 mi (48.3 km) radius of Hagerstown. The five geographic groups were stratified by study number within each zip code and the first 400 persons in each zip code area were assigned to receive one of four incentives: a pencil with the research unit's name, a copy of a newspaper article which described the study and its importance, both the article and pencil, and no incentive. A total of 2,000 persons were included in the study. Assignment to an incentive group was based on the terminal digit of the house number to eliminate the possibility that persons residing in the same household would get different incentives.

Questionnaires were sent out by first class metered mail during July along with an introductory letter, a postage-paid self-addressed envelope, and the designated incentive. Returned questionnaires with address corrections were mailed to the updated address. The questionnaires returned as non-deliverable (n = 92) were excluded from the analysis. The date of return of the questionnaires was logged into a computer data base each day. The cutoff period for the purpose of this study was also 21 days after the initial mailing.

The sample for the third study was drawn from respondents from the second study group used to test the effectiveness of mailed incentives in increasing response. This third study was conducted to test the effectiveness of a postcard reminder versus a letter plus a second questionnaire as a follow-up technique to increase subsequent response. Based on the terminal digit of their study identification number, half of the 812 nonrespondents were assigned to be sent a post-

card or a letter plus a second questionnaire. Seven weeks after the mailing of the initial questionnaire, a letter plus a second questionnaire was sent to 412 persons. Two weeks later (9 weeks after the initial mailing), a 8 in × 5 1/2 in (20.3 cm × 14 cm) fluorescent yellow postcard reminder was sent to 400 persons. Both reminders were sent by bulk mail. The long interval between initial and subsequent mailings was largely the result of printing difficulties.

RESULTS

The total initial response for the first study of questionnaire length was only 34 percent, which was not unexpected since these persons were not part of the community and most had participated just to get a free cholesterol test. The groups of respondents were equally distributed in regard to age, sex, and education. Overall, response was higher among persons aged 65–74 years (46 percent), and among those with ≥13 years of education (38 percent).

Table 1 shows the response calculated for the short and long questionnaire groups and by participant age, sex, and years of education. Twenty-one days after the initial mailing, the response for the group that was mailed the long questionnaire was 33 percent, whereas the response for the group mailed the shortened version of the questionnaire was 37 percent. There was no significant difference in responses between the two groups ($p = 0.145$). The highest responses were observed in the 65–74 years age group, 45 percent for the long questionnaire and 47 percent for the short one. There were no significant differences in response between the two groups by age, sex, or education.

TABLE 1. Number and percent responses, by age, sex, and education, to the short versus long questionnaire in a study of the effects of questionnaire length on response within 21 days of mailing: CLUE II blood donation campaign, Washington County, Maryland, May–November, 1989

Variable	No. in sample		Response (%)	
	Long questionnaire	Short questionnaire	Long questionnaire	Short questionnaire
Total	1,029	452	33.0	37.0
Age (years)				
18–24	12	10	16.7	30.0
25–34	75	38	16.0	18.4
35–44	147	75	21.1	34.7
45–54	199	84	32.2	39.3
55–64	244	95	36.5	35.8
65–74	230	105	45.2	46.7
≥75	122	45	31.2	33.3
Sex				
Male	530	242	32.1	36.4
Female	499	210	34.1	37.6
Education (years)				
<8	53	23	28.3	26.1
9–11	99	39	27.3	23.1
12	389	172	29.8	37.8
≥13	488	218	37.3	39.9

In the study of the effects of non-monetary incentives on response to a mailed questionnaire, the four incentive groups were similar in age, sex, education, and geographic location. As shown in table 2, age and education were associated with response, with the highest response being among older persons in the 65–74 years age group, and among those with ≥13 years of education. Response was significantly higher among persons who resided within Washington County (56 percent) compared with persons who lived in the rest of Maryland or West Virginia (35 percent), and Pennsylvania (29 percent). It is presumed that there was a poorer response from persons living outside of Washington County because nonresidents of the county were more likely to have come for the free cholesterol test and were less interested in the research purposes of the CLUE II program. Three weeks after the mailing, there were no appreciable differences in response among the four incentive groups (table 2).

The two groups in the third study of cost-effective techniques for the follow-up of respondents were comparable in age, sex, and years of education (table 3). Three weeks after mailing, response was significantly higher among persons who were sent a second questionnaire (23 percent) compared with 10 percent for persons who were sent only the postcard reminder. Response was highest in the older age groups and among the better educated who received a second questionnaire. In virtually all subgroups by age, sex, and education, the sending of a second questionnaire elicited a higher proportion of responses.

DISCUSSION

Questionnaire length is an important concern in epidemiologic studies because of the perceived effect on response. Researchers must balance cost and the desire to obtain optimal response, against sacrificing crucial research information. Shorter questionnaires are often recommended because it is generally believed that they result in higher frequencies of response. Evidence from other studies is inconclusive. Four of seven studies found no differences in response between long and short questionnaires (6). One study reported a 28 percent higher proportion of returns for a short questionnaire (postcard vs. two-part questionnaire), and two studies reported substantially higher returns for a longer questionnaire than for a shorter one (6). We found no evidence that reducing the

TABLE 2. Responses to incentives, by age, sex, and education, in a study of the effect of a non-monetary incentive in increasing the response to a questionnaire within 21 days of mailing: CLUE II blood donation campaign, Washington County, Maryland, May–November, 1989

Variable	Total no.	Response by incentive (%)				
		No incentive (n = 471)	Newspaper article (n = 486)	Pencil (n = 467)	Pencil and newspaper article (n = 484)	Total sample (n = 1,908)
Total	1,908	45.4	46.3	46.7	47.7	46.5
Age (years)						
18–24	18	0	25.0	33.3	50.0	33.3
25–34	133	27.0	36.1	25.9	36.4	31.6
35–44	307	34.6	34.9	27.2	35.4	32.9
45–54	409	40.6	46.2	42.9	37.7	41.8
55–64	362	52.1	39.8	55.0	52.9	50.0
65–74	434	58.5	63.9	59.3	65.1	61.8
≥75	245	50.0	41.7	54.4	50.8	48.6
Sex						
Male	760	43.2	47.5	42.6	45.1	44.6
Female	1,148	47.0	45.4	49.6	49.2	47.8
Education (years)						
<8	120	48.3	31.4	43.3	50.0	42.5
9–11	229	41.5	38.7	34.0	31.3	36.2
12	881	47.9	42.7	49.5	50.0	47.6
≥13	678	43.1	56.7	47.3	50.6	49.4

length of the questionnaire improves response enough to justify the loss of information.

The overall response in the study of the effect of a simple non-monetary incentive was 47 percent within 21 days of mailing without follow-up. The non-monetary incentives that were used had no impact on response. This finding supports the findings of others in that inclusion of non-monetary incentives can be costly and usually do not reap the expected benefits (6). An exception that influences the choice of incentives in this trial was a study of survey response among physicians, in which respondents were sent a second questionnaire alone or a second questionnaire with a pencil. Although this was a small study, enclosing a pencil produced twice the number of returns (7).

In the study of follow-up techniques, we speculate that the significant difference in response to the second questionnaire may be due to a lost or misplaced first questionnaire, especially since the time between initial and second mailings was so long. A potential respondent with a second questionnaire in hand would seem more likely to complete it rather than to search for the first questionnaire. However, a postcard may prove to be as effective if follow-up occurs within a short interval. Eighty-two percent of responses within the 21-day period after mailing in our sample occurred within 2 weeks of mailing. During this short interval, the initial nonrespondents may not have had time to misplace the first questionnaire. In the present study, delay in getting the postcards mailed due to an unforeseen printing problem probably decreased their effectiveness. In a Health Lifestyle survey in Great Britain, follow-up occurred 3 weeks after the initial mailing, and the postcard reminder was as effective as the questionnaire reminder (8). Because it is expensive to send an unneeded questionnaire, response may be increased effectively and economically by sending two reminders—first a postcard and then a second questionnaire (8).

Although various studies have been conducted to evaluate methods of increasing response, few techniques have been shown to increase response consistently. In part, this may be due to the fact that response is influenced by a variety of factors such as respondent motivation, differences in survey instruments, study design, and other characteristics which are unique to the study group. Although knowledge continues to be acquired in this area, a set of unrelated techniques that work in one study population may not be generalizable to the general population (4). Not enough has been done to show which techniques are generalizable. More important is the systematic testing of marketing principles to the specific problem of increasing response to the mailed questionnaire. These principles

TABLE 3. Number and percent responses to postcard versus second questionnaire, by age, sex, and education, in a study of follow-up techniques of nonrespondents: CLUE II blood donation campaign, Washington County, Maryland, May–November, 1989

Variable	No. in sample		Response (%)	
	Postcard	2nd questionnaire	Postcard	2nd questionnaire
Total	400	412	9.8	22.9*
Age (years)				
18–24	4	7	25.0	14.3
25–34	37	42	5.4	14.3
35–44	87	86	5.8	15.1*
45–54	86	101	11.6	28.7*
55–64	77	63	13.0	22.2
65–74	65	57	10.8	29.8*
≥75	44	56	9.1	25.0*
Sex				
Male	163	181	7.4	19.3*
Female	237	231	11.4	25.5*
Education (years)				
<8	32	28	3.1	25.0*
9–11	65	57	9.2	15.8
12	179	196	12.9	22.5*
≥13	124	131	7.3	26.0*

* $p < 0.05$.

should be tested in controlled trials, especially in pilot studies. The results should be published so that consistently successful techniques may be incorporated into new studies. This practice would lead to a better understanding of respondent behavior and to increased response to mailed questionnaires.

ACKNOWLEDGMENTS

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Clue II Active Follow-up **Health Letter**

JOHNS HOPKINS RESEARCH CENTER February, 1998

Thank You, Clue II Participants

The Johns Hopkins Research Center staff would like to thank you for taking part in the Clue II program in 1989 and again in 1996. You and the other 32,897 participants have helped greatly in our continuing efforts to solve some of the puzzles of cancer and heart disease. Using serum samples from the CLUE programs of 1974 and 1989, we have had 49 papers published in medical journals. Some of the most exciting findings of the past year are:

- Persons with low serum concentrations of beta-carotene and alpha-tocopherol in 1974 were at higher risk of developing rheumatoid arthritis than persons with high concentrations. Although this was a small study, it gains credence because it duplicated the findings of another small study in Finland. The safest recommendation at the moment is to eat fruits and vegetables rather than to take supplements.
- We have led the efforts to find other protective substances in fruits and vegetables. Some, especially some carotenoids, offer hope of being protective against cancer. In a recent study using CLUE serum samples it was found that beta-carotene is probably just a marker for other protective factors against lung cancer.

Our major research activities at present involve studies of breast and prostate cancer. The Research Center has been funded by the Department of Defense in response to the National Breast Cancer Coalition's effort to increase breast cancer research. The blood sample and health information will be used to investigate environmental and behavioral factors which may be associated with the development of breast cancer. We have also been funded by the National Cancer Institute to examine the association between exposure to pesticides and the development of prostate cancer.

The information you have provided has enabled us to continue this valuable research. We have carefully reviewed all the items that might be related to the development of disease to find what information appears most promising in the near future. After much consultation, we have developed a new questionnaire to obtain information about diet, exercise and other risk factors for cancer and heart disease. This questionnaire will be mailed in early March of 1998. We have changed this questionnaire to make it easier to read and complete. Your response will help us solve some of the puzzles of cancer and heart disease. With your continued help more pieces will be tried, some will fit, and the goal will come closer. So when your questionnaire arrives, please spare a little time to give us some more clues.

Aspirin Turns 100

August 10, 1997 marked the 100th anniversary of aspirin - one of the most widely used medications in the world. In 1897 Felix Hoffmann, a German chemist, first synthesized aspirin in a commercially producible form. Salicylic acid, the precursor for aspirin, is found in willow bark and other plants and had been used since the time of Hypocrites (400 BC) for the treatment of fever, pain, and inflammation.

Aspirin is a member of the class of compounds known as non-steroidal anti-inflammatory drugs (NSAIDS). Ibuprofen (Advil) and sodium naproxen (Aleve) are also in this class. NSAIDs are used to reduce fever, inflammation, and the pain associated with injury, menstrual cramps, arthritis and other conditions. Aspirin inhibits the blood clotting process by preventing the aggregation (clumping) of the blood cells involved in clotting.

Americans consume an estimated 40 tons of aspirin daily. Among the Clue II follow-up survey participants 49 percent of the women and 62 percent of the men reported using aspirin. Twenty-eight percent of the men and 18 percent of the women reported taking one or more tablets daily.

Continuing research into aspirin and the other NSAIDs has led to new applications for these drugs.

- Taking one aspirin at the first sign of a heart attack reduces the risk of death. Aspirin therapy is also useful in preventing heart attacks and the reoccurrence of a stroke or mini-stroke (TIA) and in preventing blood clots following coronary bypass surgery or angioplasty.
- A recent Johns Hopkins study found a lower risk of Alzheimer's disease among those who took aspirin and other NSAIDs.
- Several studies have shown a 40-50 % reduction in cancer of the rectum and colon among those who regularly took aspirin and other NSAIDs.

Whether all healthy individuals should take aspirin to prevent car-

continued on page 3

Colorectal Cancer

Colorectal cancer is the third most diagnosed cancer, and the second leading cause of cancer death in the United States. If it is detected early and treated immediately, colorectal cancer is one of the most curable kinds of cancer. Among Clue II follow-up participants 149 (1 percent) reported having colorectal cancer. The average age at diagnosis for men was 63 years and 60 years for women.

Colon cancer begins as small benign growths known as polyps. Reducing death from colorectal cancer depends on detecting and removing polyps before they become cancerous and on early treatment once cancer develops. Three screening tests are currently available. The Fecal Occult Blood Test detects blood in the stool. Sigmoidoscopy uses a flexible, hollow, lighted tube to look at the wall of the rectum and colon. The Digital Rectal Exam, the most commonly used procedure, detects tumors only within 4 inches of the anus. Current American Cancer Society recommendations for routine screening are:

- Fecal Occult Blood Test-annually after age 50
- Sigmoidoscopy-every 3 to 5 years after age 50
- Digital Rectal Exam-annually after age 40

Results from the Clue II follow-up survey demonstrate that participants are aware of the importance of colon cancer screening. One-third of participants over age 50 reported having had a sigmoidoscopy.

Among the risks factors for colorectal cancer are older age, family history of the disease, prior cancer of the endometrium, ovary, or breast, and a history of ulcerative colitis or colon polyps. Large differences in colon cancer rates among countries point to diet as another important risk factor for colon cancer. People who eat diets that are high in fiber, fruits, and vegetables and low in fat and meat tend to have a lower risk of colon cancer. The risk of colon cancer may also be reduced by non-steroidal anti-inflammatory drugs, increased physical activity, and by weight control. If you are concerned about colorectal cancer, have a health checkup and talk to your physician.

Osteoporosis

Osteoporosis is a skeletal condition in which the bones become brittle and more susceptible to fractures. Often osteoporosis is not diagnosed until a bone is fractured-usually the hip, spine, or wrist. An estimated 25 million Americans are affected by osteoporosis at a cost of 5-10 billion dollars annually. In the Clue II follow-up survey 12 percent of women age 50 or over reported a diagnosis of osteoporosis. The lifetime risk of fractures due to osteoporosis is nearly four times higher in women (40 percent) than men (13 percent).

Osteoporosis is caused by the loss of bone mineral mass which occurs with aging. For both men and women a gradual decline in bone mass begins around age 30. In women the decline in the hormone estrogen during menopause accelerates the bone loss and the effects of the disease begin to appear around ages 55-60. Men are affected by osteoporosis approximately 5 years later in life than women are. Factors which increase risk for osteoporosis are:

- advanced age
- menopause in women
- low level of testosterone in men
- low calcium diet
- family history of osteoporosis
- low level of physical activity
- cigarette smoking
- excessive alcohol consumption
- certain medications
- anorexia nervosa or bulimia
- small, thin frame

Osteoporosis can be diagnosed before a fracture occurs with a Bone Density Test. Treatment options for osteoporosis include hormone replacement therapy for postmenopausal women, calcium and vitamin D supplementation and bone building medications such as calcitonin.

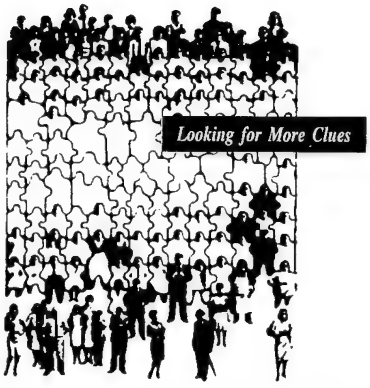
Prevention of osteoporosis begins early in life by practicing good nutrition and engaging in weight bearing exercise in order to build bone density and strength. Calcium, a mineral required for bone growth and density, is the most important dietary requirement in preventing osteoporosis. Vitamin D, which helps the body absorb and utilize calcium, is also important. For healthy men and women over 50 the recommended amount of calcium per day is 1,200 milligrams. Important dietary sources of calcium are dairy products, tofu, green leafy vegetables, and calcium fortified cereals and juices. Supplements such as calcium tablets are often used to obtain the recommended calcium intake for older individuals. Before taking any nutritional supplement consult with your doctor.

Calcium Content of Common Foods and Supplements in Milligrams

Broccoli, 1 stalk	150
Kale, 1/2 cup	103
Celery	50
Orange	50
Grapes	25
Squash, 1/2 cup	40
Whole wheat bread	22
Canned salmon	167
Tofu, 4 oz.	145
1% milk, 8 oz.	297
Cheddar cheese, 1 oz.	204
Cottage cheese, 1/2 cup	63
Yogurt, low fat	389
Cheese pizza	332
Chile, 1 cup	82
Taco, beef	174
Centrum	162
TUMS	400

Aspirin, continued

diovascular disease is still a subject for research. Aspirin can occasionally have side effects which must be weighed against the potential benefits. Aspirin can interfere with other medications, and should not be used by children and those with certain medical conditions. Consult with your doctor before beginning a regimen of aspirin or any other medication.



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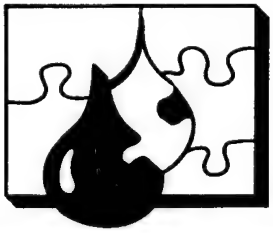


OPERATION CLUE II

Give Us A Clue

Campaign Against Cancer and Heart Disease

Clue II Active Follow-up

Health Letter

JOHNS HOPKINS RESEARCH CENTER

MARCH 2000

THE SEARCH CONTINUES.....

Thank you for taking part in the Clue II program in 1989, 1996 and again in 1998. When the Clue program began in 1974 we had no idea that it would continue for over 25 years. You and the other 32,897 participants have helped greatly in our continuing efforts to solve some of the puzzles of cancer and heart disease. Using serum samples and information from the Clue programs of 1974 and 1989, we have had over 50 papers published in medical journals. Some of the most exciting findings of the past year are:

- Postmenopausal women with low serum concentrations of vitamin B12 were at higher risk of developing breast cancer than women with high concentrations. To our knowledge this is one of the first studies to examine the association between serum levels of B-vitamins and breast cancer risk. It may be that an unidentified protective factor for breast cancer associated with higher B12 concentrations might have led to the observed protective association. These findings need to be repeated before recommendations can be made. B12 is found in animal foods, especially organ meats, eggs, milk, oysters and clams.
- A study was conducted to examine the association between serum concentrations of pesticides (DDT, PCB's) and the development of breast cancer. Results of the study are reassuring. After 20 years of follow-up, exposure to relatively high concentrations of pesticides showed no evidence of contributing to an increased risk of breast cancer.

The information you have provided has enabled us to continue this valuable research. We once again have carefully reviewed all the items that might be related to the development of disease, to find what information appears most promising in the near future. After much consultation, we have developed a new questionnaire to update previous information as well as to obtain additional information about diet, medication and supplement use, health screening practices and other risk factors for cancer and heart disease. This questionnaire will be mailed in the spring of 2000.

Again, we thank you for your help in solving some of the puzzles of cancer and heart disease. As we've said before, with your continued help more pieces will be tried, some will fit, and the goal will come closer. So when your questionnaire arrives, please spare a little more time to give us some more clues. Your contributions will make a tremendous difference, and we are grateful.

Our major research activities at present involve looking at the association between exposure to pesticides and the development of prostate cancer, and an examination of the combined effect of selenium and vitamin E in protecting against prostate cancer. We are also studying the effects of the interaction of genes and the environment on health. We hope to publish these findings in the near future.

LOOKING TOWARDS THE FUTURE:

Why is it that some people smoke all their lives and rarely have health problems and others get serious illness such as cancer or heart disease? Because we all react differently to environmental factors. These differences make us more or less susceptible to beneficial or harmful exposures in the environment. Some people may be better than others at removing harmful chemicals from the body or repairing damage the chemicals may have caused to the cell structures. The removal and repair processes are done by special proteins in the body called enzymes. We may differ in how much of the enzymes are made or how rapidly the enzymes work. One way to look at the differences in the proteins is to look at the DNA. DNA is the basic building block of life and makes up the genes that, in turn, determine how much and how well our body's enzymes may work. Natural variations in our gene structure lead to differences in protein activity. By studying how, if at all, these naturally occurring genetic variations relate to health outcomes, we can begin to learn how to modify our lifestyle such that our behavior can work better with our genetic makeup and we stay healthy.

We can look at these variations in the DNA because you gave us a blood sample and permission to store all the components of the blood. This includes white blood cells, which contain the DNA. We are looking at differ-

ences in the genes that code for enzymes that can process chemicals to be less harmful, repair damage to DNA or are involved in others ways to keep cells healthy. The genes that we are studying have variations that are usually present in 10 to 50 percent of the population. These common genetic differences are called genetic polymorphisms. Since we are just beginning these studies, we do not yet know if the genes or enzymes they produce are important in protecting us from getting cancer or other diseases. By building on the results of this study and others we hope we can get more clues on how the environment and our bodies work together to keep us well. Eventually we hope to find out who most needs help to prevent disease.

As with all of our studies, all information is kept confidential. Names are not kept with blood results. Records are kept in rooms that are locked when not in use and data in our computers are not accessible through outside telephone lines, thereby making it impossible for "hackers" to obtain access to our records. In over 35 years of doing research, confidentiality has never been breached. To make information even more secure, we are obtaining a Certificate of Confidentiality, which is issued by the National Cancer Institute as an added protection of privacy, such that persons in research will not be compelled to identify any research participants in Federal, State or local civil, criminal, administrative, legislative or other proceedings. This will give added protection to ensure that only individuals associated with our team will have access to the data. We are in the process of establishing a Community Advisory Group to review research protocols that involve participants from the community, and promote community awareness of the importance of our research in improving the health of individuals. As always, if you have any questions, please feel free to call us.

NUTRITION AND CANCER "FIVE A DAY" FOR BETTER HEALTH

Cancer is currently the second leading cause of death for American adults. It is projected to be the number one cause of early death in the next century, in part because as people live longer the risk of getting cancer increases. Lifestyle is a critical factor in most forms of cancer. Although we have little control over our genetic risks for cancer, we do have a choice in deciding which lifestyle risks to take, especially with regard to diet, smoking, and alcohol intake. One-third of all cancers in the United States are associated with tobacco use. About half of all cancers of the mouth, pharynx, and larynx are associated with heavy use of alcohol. The American Cancer Society estimates that about one-third of cancer deaths in this country are due to dietary factors.

The Johns Hopkins Research Center has added a great deal to studies of diet and cancer. Research has suggested that people who eat diets with lots of fruits and vegetables have lower risks for some cancers than people who eat few of these foods. Some evidence suggests that vegetables may protect against colon and rectal cancer because of the anti-oxidant compounds they contain, or their fiber content. Studies have also suggested that fruit and vegetable consumption also decrease breast and lung cancer risk because they contain protective plant chemicals called phytochemicals. Research has suggested that lycopene, the compound found in tomatoes that makes them red, may lower the risk of prostate cancer. Lycopene is best absorbed by the body when tomatoes are cooked; tomato paste and sauce are good sources of this substance.

"Five A Day" is a national effort to encourage us to eat at least 5 servings of fruits and vegetables everyday. Sponsored by the National Cancer Institute, this message is based on research that indicated that healthy eating starts with at least 2 servings of fruits and 3 servings of vegetables everyday. Fruits and vegetables are naturally low in calories and fat, and provide fiber, vitamins and minerals. Nutritionists recommend eating a variety of fruits and vegetables, rather than relying on vitamin and mineral supplements to help protect yourself against cancer. Research studies also suggest that eating fruits and vegetables are better than relying on supplements.

A serving is:

- 1 medium fruit
- 1 cup of leafy vegetables
- ½ cup of cut-up fruit or vegetables or cooked vegetables
- ¼ cup dried fruit
- ½ cup cooked beans or peas
- ¾ cup 100% juice

Results of the 1998 Clue II follow-up questionnaire found that 15 percent of men and 22 percent of the women, who responded to the questionnaire, eat 5 or more servings of fruits or vegetables a day. This important information will allow us to make comparisons of the health of persons with high and low fruit and vegetable intake. It's easy to add 2 servings of fruits and vegetables a day. Add a low-fat salad to lunch and crunch on an apple for a snack. Take a minute during the day and count

up how many fruits and vegetables you've had. Then you can plan for the other servings during the rest of the day. If some kinds of fruits and vegetables are healthier than others, we hope to find this out.

We can't control some factors related to our risk of getting cancer, such as a genetic predisposition, but diet is important and is under our control. Why not start now, when fruits and vegetables are plentiful, to take the Five a Day Challenge?

SELENIUM, VITAMIN E AND PROSTATE CANCER

Selenium is an essential trace mineral found in the soil. The selenium content of food varies with the concentration present in the soil. It is found in grain products, meats, eggs and shellfish. Selenium is an anti-oxidant and interacts with vitamin E which also functions as an anti-oxidant and is found most abundantly in plant oils. Together they protect cells against free radical (oxidizing) compounds. Free radicals are highly reactive compounds containing an unpaired electron when formed within cells, then cause damage and are believed to be associated with the development of disease. Free radicals seek electrons by attacking other compounds. One reason that free radicals are destructive to cells is that they can set off a chain reaction in which thousands of free radicals are generated within minutes starting from a single one. Vitamin E can stop such chain reactions and help prevent cell destruction. Vitamin E and selenium work together to protect cells against cell damage due to free radicals. Studies are underway to help clarify the combined effect of selenium and vitamin E against cancer.

A number of epidemiologic studies have found that higher blood concentrations of selenium are associated with a reduced risk of cancer. In one study among Finnish smokers, there was no association of selenium intake with subsequent prostate cancer among the groups not given vitamin E; however, among those given vitamin E, higher intakes of selenium were associated with a slightly reduced risk of prostate cancer. Selenium was not found to be effective in preventing the recurrence of skin cancers in one study. However persons taking the selenium had fewer cases of cancers of the lung, colon and rectum, and prostate, with the strongest and most significant protection being against prostate cancer. Shortly after the report of this study, another study among health professionals also found a reduced risk of prostate cancer associated with higher concentrations of selenium in toenail clippings.

In order to further investigate these findings, researchers at the Johns Hopkins Research Center have recently completed a study looking at the association of selenium in toenail clippings and vitamin E levels in the blood. In addition to the toenail and blood samples, we are also using the questionnaire information that you have provided in 1989 and during the past 3 years.

Findings from this study showed that persons with high levels of one kind of vitamin E (gamma-tocopherol) in the blood were associated with lower risk of prostate cancer, especially if these persons also had high levels of selenium. The major source of gamma-tocopherol is the diet; vitamin E supplements usually do not contain this form of vitamin E. This finding appears to be an important clue. However, like all studies among human beings, and contrary to most media stories, a single study should never be accepted as proof. If this clue is confirmed by others, then we can be reasonably certain that it fits the puzzle of prostate cancer. For the present, it is important to keep in mind that our diets can provide many essential food elements that cannot be put into pills.

Food Sources of Selenium

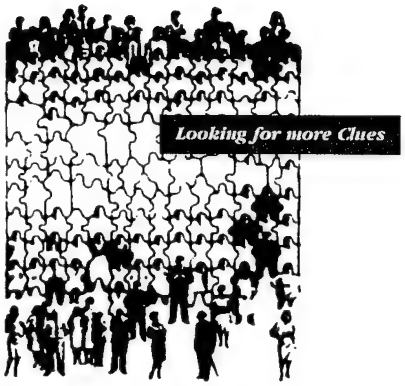
Food item and amount	Selenium (micrograms)
Canned tuna, 3 oz	68
Sirloin steak, 5 oz	48
Shrimp, 4 oz	45
Cooked egg noodles, 1 cup	35
Roasted ham, 3 oz	30
Roasted chicken, 3 oz	24
Boiled egg, 1	11
Whole-wheat bread 1 slice	10
Oatmeal, ½ cup	10
White bread, 1 slice	8

Food Sources of Vitamin E

Food item and amount	Vitamin E (IU)
Sunflower seeds, 1 oz.	21
Sunflower oil, 1 tbsp	10
Almonds, 1 oz.	10
Safflower oil, 1 tbsp	9
Wheat germ, ¼ cup	8
Peanut butter, 2 tbsp	5
Mayonnaise, 1 tbsp	1

GINKGO BILOBA RESEARCH STUDY

Washington County has been selected by the National Institute of Health as one of three sites to study the effects of the widely used herb, ginkgo biloba, in preventing or delaying changes in memory, mental alertness, and personality, that can occur in people as they get older. Ginkgo biloba (also known as the Maidenhair Tree) is derived from the leaves of one of the world's oldest surviving tree species, believed to have originated over 200 million years ago. Ginkgo



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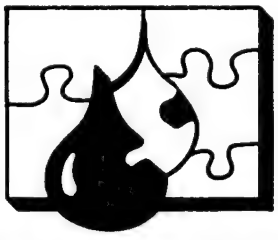
HAGERSTOWN, MD 21742-2067

trees are tall hardy trees with distinctive fan-shaped leaves. Ginkgo is considered a sacred tree by the Chinese, with medicinal use dating to ancient times.

The Cardiovascular Health Study (CHS) in Hagerstown, which is managed by Johns Hopkins University, is one of the sites funded to conduct the clinical trial. CHS is recruiting 750 men and women 75 years of age or older, who are residents of Washington County, and able to come to the CHS clinic downtown, to participate in the study. Other eligibility criteria may also apply, but will be discussed at the time of application.

We invite you to help with this important new study which will enable us to determine the value of Ginkgo biloba in the prevention of dementia, or in the delay of onset, or progression of memory loss. If you are interested in participating, please call Pat Crowley at the CHS office (301-733-8860) between the hours of 9 AM and 4 PM, and ask for the Ginkgo Biloba Trial. Participation in this study will contribute greatly to the public health.



OPERATION
CLUE II

Give Us A Clue

Campaign Against Cancer and Heart Disease

Attachment 4
1996

Study Number_____

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INTRODUCTION

Please follow the instructions carefully and answer all the questions unless otherwise instructed. Then return the completed questionnaire in the stamped envelope provided. All of the information you provide is confidential.

Thank you very much for your valuable help with this research.

INSTRUCTIONS

1. **Read each question carefully. Then use a no. 2 pencil to answer by filling in the blank space and darkening the circles.**

   Example: What is your date of birth?
   (Write in date as shown)

MONTH		DAY		YEAR	
0	1	0	8	3	0

MARKING INSTRUCTIONS

Use a No. 2 pencil only.
Make heavy black marks that darken the circle completely.
If you change your mind, please erase completely.

USE NO. 2 PENCIL ONLY

Correct Marks ● ● ● ●

Incorrect Marks ✓ ✗ ◐ ◑

2. **Unless the instructions tell you otherwise, darken only one circle.**

3. **Some questions have instructions next to the answer telling you to skip questions which do not apply to you. First darken the circle. Then follow the skip as directed.**

PLEASE CHECK THE INFORMATION BELOW AND CORRECT THE INFORMATION IF THERE IS A MISTAKE.

DO NOT MARK IN THIS AREA

THANK YOU!

If you have any questions, please feel free to call our office at (301) 791-3230

CURRENT HEALTH STATUS

1. What is your date of birth?
(Write in number and darken circles)

MONTH		DAY		YEAR	
0	0	0	0	0	0
1	1	1	1	1	1
	2	2	2	2	2
	3	3	3	3	3
	4		4	4	4
	5		5	5	5
	6		6	6	6
	7		7	7	7
	8		8	8	8
	9		9	9	9

2. How much do you weigh?
(Write in number and darken circles)

POUNDS		
0	0	0
1	1	1
2	2	2
3	3	3
4	4	4
5	5	5
	6	6
	7	7
	8	8
	9	9

3. What is your marital status?

- ○ Never married
- ○ Married
- ○ Widowed
- ○ Divorced
- ○ Separated

4. After you were 21 years old, about how many times have you GAINED AND LOST 10 or more pounds? (NOT counting PREGNANCY and NURSING.)

- ○ Never
- ○ 1-5 times
- ○ 6-10 times
- ○ 11-15 times
- ○ 16 or more times

HEALTH HISTORY

5. Have you ever been told by a doctor or other health professional that you have any of the conditions listed below?

	No	Yes	How old were you when you were first told you had this condition?
a. Diabetes	○	○	_____
b. High cholesterol	○	○	_____
c. Heart attack	○	○	_____
d. Angina pectoris	○	○	_____
e. Stroke	○	○	_____
f. TIA (transient ischemic attack)	○	○	_____
g. Peripheral artery disease or claudication of legs (pain with walking or exercise) (not varicose veins)	○	○	_____
h. Osteoporosis	○	○	_____
i. Hip fractures	○	○	_____
j. Wrist or Colles' fracture	○	○	_____
k. Fibrocystic disease of the breast or other benign breast disease	○	○	_____
l. Endometriosis	○	○	_____
m. Uterine fibroids	○	○	_____
n. High blood pressure (excluding during pregnancy)	○	○	_____
o. Migraine headaches	○	○	_____
p. Thyroid disease	○	○	_____
q. Rheumatoid arthritis	○	○	_____
r. Gallbladder disease	○	○	_____
s. Gastric or duodenal ulcer	○	○	_____
t. Macular degeneration of the retina	○	○	_____

Question 5 continued from page 2	No	Yes	How old were you when you were first told you had this condition?
u. Cataract	○	○	_____
v. Asthma	○	○	_____
w. Emphysema or chronic bronchitis	○	○	_____
x. Diverticulitis/diverticulosis	○	○	_____
y. Parkinson's disease	○	○	_____
z. Kidney stones	○	○	_____
aa. Ulcerative colitis/Crohn's disease	○	○	_____
bb. Breast cancer	○	○	_____
cc. Cancer of the cervix (Include in-situ)	○	○	_____
dd. Cancer of the uterus (endometrium)	○	○	_____
ee. Cancer of the ovary	○	○	_____
ff. Colon or rectal polyp (benign)	○	○	_____
gg. Cancer of the colon or rectum	○	○	_____
hh. Cancer of the lung	○	○	_____
ii. Melanoma	○	○	_____
jj. Basal cell skin cancer	○	○	_____
kk. Squamous cell skin cancer	○	○	_____
ll. Prostate cancer	○	○	_____
mm. Other cancer (Specify site of other cancer)	○	○	_____
nn. Other major illness (specify illness)	○	○	_____ _____ _____

6. Have you ever had any of the following surgical operations?

Operation or Surgery	How old were you when you had this surgery?	Where was this surgery done?
BREAST BIOPSY OR LUMPECTOMY (removal of breast tissue) ○ No ○ Yes If yes, number of biopsies or lumpectomies ○ One ○ Two ○ Three or more How old were you when you had your MOST RECENT BIOPSY? Did the biopsies show breast cancer? ○ No ○ Yes	_____ Years old	○ Washington County Hospital ○ Other, specify hospital, city, state: _____ _____
MASTECTOMY (removal of a breast) ○ No ○ Yes If yes, how many breasts were removed? ○ One breast ○ Both breasts	_____ Years old	○ Washington County Hospital ○ Other, specify hospital, city, state: _____ _____

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DO NOT MARK IN THIS AREA

6. Have you ever had any of the following surgical operations? (Continued)

Operation or Surgery	How old were you when you had this surgery?	Where was this surgery done?
HYSTERECTOMY (removal of uterus) ○ No ○ Yes	_____ Years old	○ Washington County Hospital ○ Other, specify hospital, city, state: _____ _____
OOPHORECTOMY (removal of ovaries) ○ No ○ Yes ○ One ovary ○ Both ovaries	_____ Years old	○ Washington County Hospital ○ Other, specify hospital, city, state: _____ _____
PROSTATE SURGERY ○ No ○ Yes If yes, what type of surgery? Transurethral Resection (TURP) ○ No ○ Yes Biopsy ○ No ○ Yes Prostatectomy (removal of the prostate gland) ○ No ○ Yes	_____ Years old _____ Years old _____ Years old	○ Washington County Hospital ○ Other, specify hospital, city, state: _____ _____
VASECTOMY (male sterilization) ○ No ○ Yes	_____ Years old	○ Washington County Hospital ○ Other, specify hospital, city, state: _____ _____
OTHER SURGERIES ○ No ○ Yes If yes, what other surgeries have you had? _____ _____ _____	_____ Years old _____ Years old _____ Years old	○ Washington County Hospital ○ Other _____ ○ Washington County Hospital ○ Other _____ ○ Washington County Hospital ○ Other _____

FAMILY HISTORY

7. Have any of the following blood-related relatives ever had cancer?(Do not count foster or step parents)

Relative	Ever had cancer			Type(s) of cancer	Age when cancer was found
Mother	○ No	○ Yes	○ Don't know	_____	_____ Years old
Mother's Mother	○ No	○ Yes	○ Don't know	_____	_____ Years old
Mother's Father	○ No	○ Yes	○ Don't know	_____	_____ Years old
Father	○ No	○ Yes	○ Don't know	_____	_____ Years old
Father's Mother	○ No	○ Yes	○ Don't know	_____	_____ Years old
Father's Father	○ No	○ Yes	○ Don't know	_____	_____ Years old

8. How many blood related SISTERS do you have?

○ None (Go to Question 11) ○ Two ○ Four ○ Six or more
○ One ○ Three ○ Five

9. How many of your blood related SISTERS ever had cancer?

○ None (Go to Question 11) ○ Two ○ Four ○ Six or more
○ One ○ Three ○ Five ○ Don't know

10. For each of your blood related SISTERS who ever had cancer, please list the type(s) of cancer and how old your SISTER was when the cancer was found.

Sister #	Type(s) of cancer	Age when cancer was found
○ 1	_____	_____ years old
○ 2	_____	_____ years old
○ 3	_____	_____ years old

11. How many blood related BROTHERS do you have?

○ None (Go to Question 14) ○ Two ○ Four ○ Six or more
○ One ○ Three ○ Five

12. How many of your blood related BROTHERS ever had cancer?

○ None (Go to Question 14) ○ Two ○ Four ○ Six or more
○ One ○ Three ○ Five ○ Don't know

13. For each of your blood related BROTHERS who ever had cancer, please list the type(s) of cancer and how old your BROTHER was when the cancer was found.

Brother #	Type(s) of cancer	Age when cancer was found
○ 1	_____	_____ years old
○ 2	_____	_____ years old
○ 3	_____	_____ years old

14. How many blood related CHILDREN do you have? (Do not count, adopted, foster, or step children)

○ None (Go to Question 17) ○ Two ○ Four ○ Six or more
○ One ○ Three ○ Five

15. How many of your blood related CHILDREN ever had cancer?

○ None (Go to Question 17) ○ Two ○ Four ○ Six or more
○ One ○ Three ○ Five ○ Don't know

16. For each of your blood related CHILDREN who ever had cancer, please list the type(s) of cancer and how old your CHILD was when the cancer was found.

Child #	Type(s) of cancer	Age when cancer was found
○ 1	_____	_____ years old
○ 2	_____	_____ years old
○ 3	_____	_____ years old

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DO NOT MARK IN THIS AREA

17. How often on average have you taken any of the following medications?

MEDICATION	NUMBER OF PILLS TAKEN								DOSE / STRENGTH				DURATION: How many years taken?							Are you taking now?	
	None	Less than 1 per week	1-3 per week	4-6 per week	1 per day	2 per day	3 per day	4 or more per day	Baby	Regular (325mg)	Extra Strength (500 mg)	Don't Know	1	2-4	5-9	10-14	15-19	20-24	25+	NO	YES
Acetaminophin (for example, Tylenol, Anacin-3 or other brands)	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Aspirin (for example, Bufferin, AlkaSeltzer, Bayer, Ecotrin or other brands)	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Other Anti-Inflammatory (for example Ibuprofen, Advil, Ibuprin, Motrin, Excedrin IB, Aleve, Ansaid, Naproxen, Naprosyn, Sulindac, Orudis, Piroxicam, Nalfon, or other brands) Usual Brand: ____ ____ ____	○	○	○	○	○	○	○	○					○	○	○	○	○	○	○	○	○
Melatonin	○	○	○	○	○	○	○	○					○	○	○	○	○	○	○	○	○

18. Have you ever taken any MULTIPLE vitamins or minerals regularly (at least once per week)?

○ No (Go to Question 19) ○ Yes (Please complete the following):

Brand Name	How many Years taken?							Usual Number of Tablets or Capsules taken						Are you taking now?	
	1or less	2-4	5-9	10-14	15-19	20-24	25+	Less Than 1 per week	One per week	2-3 per week	4-6 per week	1 per day	More than 1 per day	No	Yes
____	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
____	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
____	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
____	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
____	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
____	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
____	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
____	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○

19. Have you ever taken any INDIVIDUAL vitamin or INDIVIDUAL mineral regularly (at least once per week)?

○ No (Go to Question 20) ○ Yes (Please complete the following):

Name of INDIVIDUAL Vitamin or Mineral	Dose per tablet or capsule	How many Years INDIVIDUAL VITAMIN OR MINERAL taken?							Usual Number of Tablets or Capsules taken						Are you taking now?	
		1or less	2-4	5-9	10-14	15-19	20-24	25+	Less Than 1 per week	One per week	2-3 per week	4-6 per week	1 per day	More than 1 per day	No	Yes
Example Vitamin C	400 mg.															
		○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
		○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
		○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
		○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
		○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
		○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
		○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
		○	○	○	○	○	○	○	○	○	○	○	○	○	○	○

20. Since 1989, have you had a colonoscopy or sigmoidoscopy (examination of the colon)?

○ NO ○ YES

21. Have you and your spouse (or partner) ever tried to become PREGNANT for more than one year without success?

○ NO (Go to Question 26) ○ YES ○ NOT APPLICABLE (Go to Question 26)

22. What was the cause of infertility? Mark all that apply.

- ○ Did not look for cause
- ○ Tubal blockage
- ○ Ovulation / hormonal problem
- ○ Endometriosis
- ○ Cervical mucus factor
- ○ Cause not found
- ○ Male infertility
- ○ Other, specify________

23. Were you treated for infertility?

○ NO (Go to Question 26) ○ YES

24. What treatments did you have for infertility? Mark all that apply.

- ○ Surgery
- ○ Pergonal
- ○ Chlomid
- ○ Other, specify________

25. Were the treatments successful?

○ NO ○ YES ○ DON'T KNOW

30028

DO NOT MARK IN THIS AREA

26. Have you ever used any of the following?

- ○ Cigars
- ○ Pipes
- ○ Snuff
- ○ Chewing tobacco
- ○ Cigarettes
- ○ None

If you've never smoked cigarettes, go to question 31.

27. At what ages were you smoking? (Complete all that apply)

- ○ 5 - 14 years old
- ○ 15 - 24 years old
- ○ 25 - 34 years old
- ○ 35 - 44 years old
- ○ 45 - 54 years old
- ○ 55 - 64 years old
- ○ 65 years or older

Total number of years smoked cigarettes_____

28. How many cigarettes do you or did you usually smoke each day?

- ○ Less than 1 per day
- ○ 1-4
- ○ 5-14
- ○ 15-24
- ○ 25-34
- ○ 35 or more

29. Have you ever stopped smoking for 6 months or more?

- ○ NO (Go to Question 31)
- ○ YES

30. If YES, how many times have you stopped smoking for 6 months or more?

- ○ Once
- ○ Twice
- ○ Three times
- ○ Four times or more

31. Before you were 21 years old, how many years did you work in the same room or live with someone who smoked cigarettes?

Number of years (Write in number and darken circles)

○ 0	0
○ 1	1
○ 2	2
○ 3	3
○ 4	4
○ 5	5
○ 6	6
	7
	8
	9

32. After you were 21 years old, how many years did you work in the same room or live with someone who smoked cigarettes?

Number of years (Write in number and darken circles)

○ 0	0
○ 1	1
○ 2	2
○ 3	3
○ 4	4
○ 5	5
○ 6	6
○ 7	7
○ 8	8
○ 9	9

33. When you lived or worked with someone who smoked cigarettes, on average how many hours per day were you exposed to someone else's smoke?

- ○ 0 Hours
- ○ 1 - 3 Hours
- ○ 4 - 6
- ○ 7 - 10
- ○ 11 - 16
- ○ More than 17 hours

34. **Have you ever drunk alcoholic beverages (such as beer, wine or liquor) at least once a month?**

○ No (Go to Question 40) ○ Yes

35. **How old were you when you FIRST STARTED drinking alcoholic beverages at least once a month?**

Years Old (Write in number and darken circles)

⓪	0
①	1
②	2
③	3
④	4
⑤	5
⑥	6
⑦	7
	8
	9

36. **Do you drink alcoholic beverages at least once a month NOW?**

○ No ○ Yes

37. **Considering the time you may have stopped drinking and then restarted, how many TOTAL YEARS have you actually drunk alcohol?**

Number of years (Write in number and darken circles)

⓪	0
①	1
②	2
③	3
④	4
⑤	5
⑥	6
⑦	7
⑧	8
⑨	9

38. **How many drinks of alcoholic beverage do/did you USUALLY have PER WEEK? (Consider a drink to be a drink or shot of liquor, a 4oz. serving of wine, or one 12oz. can or bottle of beer, light beer, or a wine cooler.)**

Number of drinks per week (Write in number and darken circles)

⓪	⓪
①	①
②	②
③	③
④	④
⑤	⑤
⑥	⑥
⑦	⑦
⑧	⑧
⑨	⑨

39. **On how many DAYS do/did you drink each of the following?**

	None	1-3 days per month	1 day per week	2-3 days per week	4-6 days per week	Every day
Beer	○	○	○	○	○	○
Wine	○	○	○	○	○	○
Liquor	○	○	○	○	○	○

40. The questions in this section ask about whether, and how often, you ate certain kinds of meat during the LAST 12 MONTHS.

For each of the foods you eat, please mark how often you ate it and how it was cooked.

During the LAST 12 MONTHS or so, how often did you eat...	AVERAGE USE LAST 12 MONTHS: Never or seldom	1 per month	2-3 per month	1 per week	2 per week	3-4 per week	5-6 per week	1 per day	2+ per day	What was the one most common method of cooking?(check one)	How was it usually cooked on the outside?
A. Hamburger/ Cheeseburger?	○	○	○	○	○	○	○	○	○	○ Pan fried ○ Oven-broiled ○ Grilled/barbecued ○ Other	○ Not browned or ○ Slightly browned ○ Medium brown ○ Well browned ○ Blackened/charred
B. Beef Steak?	○	○	○	○	○	○	○	○	○	○ Pan fried ○ Oven-broiled ○ Grilled/barbecued ○ Other	○ Not browned or ○ Slightly browned ○ Medium brown ○ Well browned ○ Blackened/charred
C. Fried Chicken?	○	○	○	○	○	○	○	○	○	○ Pan fried ○ Oven-Fried ○ Deep Fried	○ Not browned or ○ Slightly browned ○ Medium brown ○ Well browned ○ Blackened/charred
D. Chicken (other than fried)?	○	○	○	○	○	○	○	○	○	○ Oven-Baked ○ Grilled/barbecued ○ Broiled ○ Other	○ Not browned or ○ Slightly browned ○ Medium brown ○ Well browned ○ Blackened/charred
E. Pork Chops?	○	○	○	○	○	○	○	○	○	○ Pan fried ○ Oven-broiled ○ Grilled/barbecued ○ Other	○ Not browned or ○ Slightly browned ○ Medium brown ○ Well browned ○ Blackened/charred
F. Bacon?	○	○	○	○	○	○	○	○	○	○ Pan fried ○ Oven-broiled ○ Grilled/barbecued ○ Other	○ Not browned or ○ Slightly browned ○ Medium brown ○ Well browned ○ Blackened/charred
G. Fish?	○	○	○	○	○	○	○	○	○	○ Pan fried ○ Oven-broiled ○ Grilled/barbecued ○ Other	○ Not browned or ○ Slightly browned ○ Medium brown ○ Well browned ○ Blackened/charred

30028

DO NOT MARK IN THIS AREA

41. When you were an infant were you breast fed or bottle fed?

() Breast fed () Bottle fed () Both breast and bottle fed () Don't Know

42. When did your hair begin to gray? By graying we mean the appearance of more than just a few gray hairs.

() Not yet
() Less than 20 years old
() 20 to 24 years old
() 25 to 29 years old
() 30 to 34 years old
() 35 to 39 years old
() 40 to 44 years old
() 45 to 49 years old
() 50 years or older

43. Have you ever used hair coloring products for more than 6 months?

() No (Men: Continue with question 47)
(Women: Continue with Question 53)
() Yes (Please complete the following)

First answer "Yes" or "No" for each type of hair color product listed. If you answered "Yes" in Column A then answer the questions in Columns B, C, D & E for that product. If answered "No" then go to the next hair coloring product.

Have you ever used this product?	At What Age Did You Start Using This Product?	How Many Years Have You Used This?	How Often Did You Apply This Coloring Product?	What Color Did You Usually Use?
44. Temporary Rinses (Color removed by 1-2 shampoos) () Yes → () No (Go to question 45)	_____Years Old	_____Years	() 1-2 times per year () 3-10 times per year () 11-20 times per year () 21-40 times per year () 40 or more times per year	() Brown () Black () Red () Blond () Silver toners () Other
45. Semi-permanent products (Color washed out by 6-12 shampoos) () Yes → () No (Go to question 46)	_____Years Old	_____Years	() 1-2 times per year () 3-10 times per year () 11-20 times per year () 21-40 times per year () 40 or more times per year	() Brown () Black () Red () Blond () Silver toners () Other
46. Permanent products (Color does not wash out when you shampoo your hair) () Yes → () No Men: (Go to question 47) Women: (Go to question 53)	_____Years Old	_____Years	() 1-2 times per year () 3-10 times per year () 11-20 times per year () 21-40 times per year () 40 or more times per year	() Brown () Black () Red () Blond () Silver toners () Other

MEN: PLEASE COMPLETE QUESTIONS 47 THRU 52
WOMEN: PLEASE GO TO QUESTION 53

47. A digital rectal exam is when a doctor inserts his finger in the rectum to check for problems such as an enlarged prostate gland or polyps.

Have you ever had a digital rectal exam?

- ○ No (Go to Question 49)
- ○ Yes (Continue with Question 48)

48. How many years has it been since your last digital rectal exam?

- ○ Less than one year
- ○ one year
- ○ two years
- ○ three or more years

49. Have you ever had a blood test (PSA) to see if you had prostate cancer?

- ○ No (Go to Question 52)
- ○ Yes (Continue with Question 50)

50. Has your PSA blood test ever been abnormal?

- ○ No (Go to Question 52)
- ○ Yes (Continue with Question 51)

51. Was it followed up by: (Mark all that apply.)

- ○ Not followed up
- ○ Ultrasound
- ○ Biopsy
- ○ Surgical operation
- ○ Radiation
- ○ Hormones

52. Have you ever taken the following hormones?

			How many years did you take the hormones?						
			1 or less	2 - 4	5 - 9	10 - 14	15 - 19	20 - 24	25 +
Thyroid Hormones	○ No	○ Yes	○	○	○	○	○	○	○
Testosterone	○ No	○ Yes	○	○	○	○	○	○	○
Anabolic Steroids	○ No	○ Yes	○	○	○	○	○	○	○

MEN: PLEASE GO TO QUESTION 77
WOMEN: PLEASE COMPLETE THE FOLLOWING

53. How old were you when you first started having MENSTRUAL PERIODS?

Age Periods Started (Write in number and darken circles)

⓪ ① ②	⓪ ① ② ③ ④ ⑤ ⑥ ⑦ ⑧ ⑨

DO NOT MARK IN THIS AREA

54. Have your MENSTRUAL PERIODS stopped permanently?

- ○ Yes, menstrual periods stopped. (Continue with Question 55)
- ○ Had menopause, but now have periods due to hormone replacement therapy. (Continue with Question 55)
- ○ No, still menstruating (Go to Question 57)
- ○ Not sure (Go to Question 57)

55. How old were you when your natural MENSTRUAL PERIODS stopped?

Age Periods Stopped (Write in number and darken circles)

⓪	0
①	1
②	2
③	3
④	4
⑤	5
	6
	7
	8
	9

56. Why did your natural MENSTRUAL PERIODS stop?

- ○ Surgical Menopause (Hysterectomy or removal of uterus)
- ○ Natural menopause (Change of Life)
- ○ Other, specify_____

57. Have you ever taken BIRTH CONTROL PILLS (oral contraceptives)?

- ○ No (Go to Question 60)
- ○ Yes (Continue with Question 58)

58. At what age did you first use birth control pills?

⓪	0
①	1
②	2
③	3
④	4
	5
	6
	7
	8
	9

(Write in number and darken circles)

59. Altogether, how many years did you take BIRTH CONTROL PILLS (oral contraceptives)?

Total Number of Years

⓪	0
①	1
②	2
③	3
	4
	5
	6
	7
	8
	9

(Write in number and darken circles)

60. Have you EVER taken ESTROGENS (hormones, such as Premarin) alone or in combination with progestin for symptoms or effects of menopause?

- ○ No (Go to Question 65)
- ○ Yes (Continue with Question 61)

61. At what age did you first use estrogens for effects of menopause?

(0)	0
(1)	1
(2)	2
(3)	3
(4)	4
(5)	5
(6)	6
(7)	7
(8)	8
(9)	9

(Write in number and darken circles)

62. Are you currently taking ESTROGENS?

- ○ No
- ○ Yes

63. Altogether, how many years did you take ESTROGENS?

Total number of years (Write in number and darken circles)

(0)	0
(1)	1
(2)	2
(3)	3
(4)	4
	5
	6
	7
	8
	9

64. What type of estrogens do/did you use?

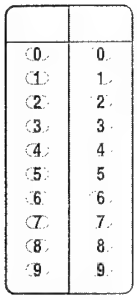
- ○ Patch
- ○ Pills
- ○ Shots
- ○ Vaginal creams or suppositories
- ○ Not sure
- ○ Other, specify:_____

65. Have you ever taken PROGESTINS (hormones such as Provera) alone or in combination with estrogens for symptoms or effects of MENOPAUSE?

- ○ No (Go to Question 69)
- ○ Yes

DO NOT MARK IN THIS AREA

66. At what age did you first use <u>PROGESTINS?</u>



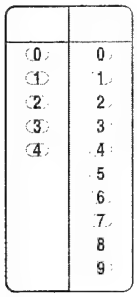
(Write in number and darken circles)

67. Are you currently taking <u>PROGESTINS?</u>

- ○ **No**
- ○ **Yes**

68. Altogether, how many years did you take <u>PROGESTINS?</u>

<u>Total Number of Years</u>



(Write in number and darken circles)

69. Have you ever taken thyroid hormones?

- ○ **No (Go to Question 71)**
- ○ **Yes (Continue with Question 70)**

70. How many years did you take thyroid hormones?

0 - 1	2 - 4	5 - 9	10 - 14	15 - 19	20 - 24	25+
○	○	○	○	○	○	○

71. Have you ever been pregnant?

- ○ **No (Go to Question 77)**
- ○ **Yes (Continue with Question 72)**

72. For each time you became pregnant, please mark the outcome of the PREGNANCY.

	PREGNANCY OUTCOME			
	Live Birth	Stillborn	Miscarriage	Abortion
1st Pregnancy	○	○	○	○
2nd Pregnancy	○	○	○	○
3rd Pregnancy	○	○	○	○
4th Pregnancy	○	○	○	○
5th Pregnancy	○	○	○	○
6th Pregnancy	○	○	○	○
7th Pregnancy	○	○	○	○
8th Pregnancy	○	○	○	○
9th Pregnancy	○	○	○	○
10th Pregnancy	○	○	○	○
11th Pregnancy	○	○	○	○

73. How old were you when your first child was born?

Age **(Write in number and darken circles)**

○	0
1	1
2	2
3	3
4	4
5	5
	6
	7
	8
	9

○ **Does not apply**

74. Are you pregnant now?

○ **No**

○ **Yes**

75. Did you breastfeed any of your children?

○ **No (Go to Question 77)**

○ **Yes (Continue with Question 76)**

76. In total, how many months of your life have you spent breast feeding?

Months **(Write in number and darken circles)**

○	0
1	1
2	2
3	3
4	4
5	5
6	6
7	7
8	8
9	9

77. PLEASE INDICATE THE NAME OF SOMEONE AT A DIFFERENT ADDRESS THAT WE MIGHT WRITE TO IN THE EVENT WE ARE UNABLE TO CONTACT YOU.

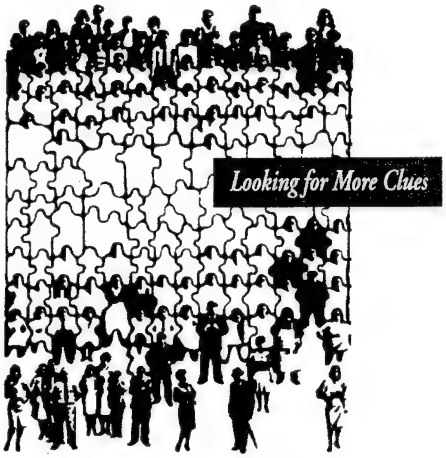
Name: _____ **Relationship:** _____
First **Middle** **Last**

Address: _____

_____

DO NOT MARK IN THIS AREA

THANK YOU FOR COMPLETING THE QUESTIONNAIRE!



STUDY NO.

0 0 0 0 0
1 1 1 1 1
2 2 2 2 2
3 3 3 3 3
4 4 4 4 4
5 5 5 5 5
6 6 6 6 6
7 7 7 7 7
8 8 8 8 8
9 9 9 9 9

CLUE II

Please check the information above and correct the information if there is a mistake.

If the person whose name appears on this form is deceased, please ***STOP HERE*** *and provide the* ***date of death***:

__/__/__
MONTH DAY YEAR

Please return the booklet in the enclosed postage-paid envelope.

INSTRUCTIONS

USE A NO. 2 PENCIL ONLY

THIS FORM IS DESIGNED TO BE READ BY OPTICAL SCANNING EQUIPMENT.

1. Please use an ordinary **NO. 2 PENCIL** to answer all questions.
2. Make heavy black marks that darken the circle *completely.*
   Please do *not* mark this way: ✓ ✗ ◒ · Please mark this way: ●
3. If you change your mind, please erase completely.
4. Unless the instructions tell you otherwise, darken only one circle.
5. Note that some questions ask for information by certain time periods and some ask for current status.

EXAMPLE

Have you been told by a doctor or other health professional that you have any of the conditions listed to the right?

Mark the "Yes" circle ***and*** *Year of First Diagnosis circle for each illness you have had diagnosed.*

Mark here for "Yes".	YEAR OF FIRST DIAGNOSIS: Before 1989	1989 to July 1, 1996	After July 1, 1996
Diabetes mellitus ●	●	○	○
Elevated cholesterol ○	○	○	○
High blood pressure ●	○	○	●

6. If you have comments, please write them on the last page of the booklet.

IF YOU HAVE ANY QUESTIONS, PLEASE FEEL FREE TO CALL OUR OFFICE AT (301) 791-3230.

PLEASE DO NOT WRITE IN THIS AREA ○●○○○●●●○○●●○●●○○○○○○○○○

0027874

1 **What is your date of birth?** ___/___/___ MONTH DAY YEAR

2 **Have you been told that you have cancer?**

	YEAR OF DIAGNOSIS		
Mark here for "Yes".	Before 1989	1989 to July 1, 1996	After July 1, 1996
○	○	○	○

If "No", go to question 5.

3 **If you had cancer, what type of cancer did you have? (Please mark below)**

TYPES OF CANCER

- ○ Bladder
- ○ Breast
- ○ Cervix
- ○ Colon or rectum
- ○ Leukemia
- ○ Lung
- ○ Lymphoma or Hodgkins
- ○ Melanoma
- ○ Ovary
- ○ Pancreas
- ○ Prostate
- ○ Skin (basal or squamous)
- ○ Uterus or endometrium
- ○ Other or unknown (Please specify) _____

Age at diagnosis of *first* cancer (excluding skin cancer):

- ○ Under 20
- ○ 20–39
- ○ 40–49
- ○ 50–59
- ○ 60–69
- ○ 70 or over

4 **Where was the cancer diagnosed?**

- ○ Washington County Hospital
- ○ Other (Please specify hospital or office and city, state) _____

5 **Have you been told by a doctor or other health professional that you have any of the conditions listed below?**

Mark here for "Yes".	YEAR OF DIAGNOSIS: Before 1989	1989 to July 1, 1996	After July 1, 1996	Where was the diagnosis made? (Please specify hospital or office and city, state.)
A. Diabetes				
B. Heart attack				
C. Angina pectoris				
D. Stroke				
E. Peripheral artery disease (pain with walking or exercise; not varicose veins)				
F. Osteoporosis				
G. Hip fracture				
H. Wrist fracture				
I. Fibrocystic disease of the breast or other benign breast disease (not cancer)				
J. Endometriosis				
K. Uterine fibroids				
L. Macular degeneration of the retina				
M. Cataracts				
N. Colon or rectal polyps (benign; not cancer)				
O. Other major illness (Specify illness)				

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6 **During the *past 10 years* (since the time you donated to CLUE II) on average, how often have you taken the following? (Please answer each item below.)**

MEDICATIONS	NUMBER OF PILLS TAKEN: Never or less than one a month	Less than one per week	1–3 per week	4–6 per week	1 per day	2 per day	3 per day	4 or more per day
Aspirin—Baby or low-dose (162 mg or less)	○	○	○	○	○	○	○	○
Regular or extra-strength aspirin—(163 mg or more) For example: Bufferin, Anacin, Bayer, Excedrin, Ecotrin, etc.	○	○	○	○	○	○	○	○
Acetaminophen—For example: Tylenol, Phenaphen, etc.	○	○	○	○	○	○	○	○
Ibuprofen—For example: Motrin, Advil, Nuprin, Mediprin, etc.	○	○	○	○	○	○	○	○
Anti-inflammatory analgesics (other than aspirin)—For example: Naprosyn, Anaprox, Aleve, Voltaren, Feldene, Toradol, Indocin, etc.	○	○	○	○	○	○	○	○

7 **During the *past 10 years* (since the time you donated to CLUE II), have you taken any of the following medications for your heart or blood pressure?**

	No	Yes, but not currently	Yes, currently
Calcium Blocker—For example: Procardia, Cardizem, Norvase, Calan, Adalat, Sudar, verapamil, amlodipine, etc.	○	○	○
Beta Blocker—For example: Lopressor, Tenormin, Inderal, atenolol, metroprolol, etc.	○	○	○
ACE Inhibitor—For example: Vasotec, Zestril, Capoten, Prinivil, Lotensin, Accupril, Monopril, captopril, etc.	○	○	○
Diuretic—For example: Lasix, Lozol, triamterene, HCTZ, furosemide, thiazides, etc.	○	○	○
Other—Mark here if unsure of name of heart or blood pressure medication category (Specify medicine)	○	○	○

8 **Do you *now* use any of the following tobacco products?**

- ○ Cigars
- ○ Cigarettes
- ○ Pipes
- ○ Snuff
- ○ Chewing tobacco
- ○ None

9 **If you smoke cigarettes, how many do you usually smoke each day at the *present* time?**

- ○ Do not smoke cigarettes
- ○ Less than 1 per day
- ○ 1–4
- ○ 5–14
- ○ 15–24
- ○ 25–34
- ○ 35 or more

10 **Have you ever used "nicotine gum" or a "nicotine patch" to try to quit smoking?**

- ○ No
- ○ Yes, nicotine gum only
- ○ Yes, nicotine patch only
- ○ Yes, both nicotine gum and nicotine patch

11 **This question asks about exercise during high school and young adulthood. During these ages, on average about how many months during the year did you take part in moderate or strenuous (aerobic) physical activity or sports at least twice per week?**

(Examples are swimming, aerobics, field hockey, basketball, cycling, and running; farm chores; brisk walking; other strenuous work)

	MONTHS PER YEAR				
	Never	Less than 4	4–6	7–9	10–12
During high school	○	○	○	○	○
During ages 18–22	○	○	○	○	○

12 **How many years (on average) over your whole life did you take part in moderate or strenuous exercise 4 or more hours per week?**

- ○ Less than 1 year
- ○ 1–5 years
- ○ 6–10 years
- ○ 11–15 years
- ○ 16–20 years
- ○ More than 20 years

SELECT THE ANSWER TO EACH QUESTION WHICH BEST DESCRIBES YOU.

13 **What is/was your *MAIN* occupation? (Include homemaker and voluntary work)**

_____

14 **Are you currently employed? (Answer only one)**

○ No ○ Yes ○ Retired (not working) ○ Retired but still working

15

Currently at work I . . .	Never	Seldom	Sometimes	Often	Always
sit	○	○	○	○	○
stand	○	○	○	○	○
walk	○	○	○	○	○
lift heavy loads	○	○	○	○	○

16

	Never	Seldom	Sometimes	Often	Very often
After working, I am tired	○	○	○	○	○
At work I sweat	○	○	○	○	○

17 **In comparison with others of my own age I think my work is physically:**

○ Much heavier ○ Heavier ○ As heavy ○ Lighter ○ Much lighter

18 For each activity that you do, please darken the circle for the number of hours per week *and* the number of months per year.

ACTIVITY	Do not do	NUMBER OF HOURS PER WEEK: Less than 1	1–2	2–3	3–4	More than 4	NUMBER OF MONTHS PER YEAR: Less than 1	1–3	4–6	7–9	More than 9
Walking or hiking (include walking to activities)											
Jogging (slower than 10 min/mile)											
Running (10 min/mile or faster)											
Bicycling (include stationary machine)											
Calisthenics/Aerobics/ Aerobic Dancing/Rowing Machine/Treadmill											
Tennis/Squash/Racquet Ball											
Swimming											
Household activities (sweeping, vacuuming, washing floor)											
Lawn work and gardening											
Other activities (Please specify) _____											

19 In comparison with others of my own age, I think my physical activity during leisure time is:

- Much less
- Less
- The same
- More
- Much more

20 During my leisure time I sweat:

- Never
- Seldom
- Sometimes
- Often
- Very often

21 During leisure time I . . .

	Never	Seldom	Some-times	Often	Always
play sports					
watch television					
walk					
bicycle					

22 How many minutes do you walk and/ or bicycle per day (for example, to and from work, school and shopping)?

- None
- Less than 5
- 5–15
- 15–30
- 30–45
- More than 45

23 What is your usual walking pace outdoors?

- Easy, casual (less than 2 mph)
- Normal, average (2–2.9 mph)
- Brisk pace (3–3.9 mph)
- Very brisk/striding (4 mph or faster)
- Unable to walk

24 How many *flights* of stairs (not individual steps) do you climb daily? (1 flight = 10 steps)

- 2 flights or less
- 3–4 flights
- 5–9 flights
- 10–14 flights
- 15 or more flights

THERE IS INTEREST IN WHETHER SLEEP PATTERNS ARE RELATED TO HEALTH. PLEASE ANSWER THE FOLLOWING QUESTIONS ABOUT YOUR SLEEP HABITS.

25 **How many hours do you usually sleep at night (or your main sleep period) on weekdays or workdays?**

- Less than 6 hours
- 6 hours
- 7 hours
- 8 hours
- 9 hours
- 10 hours
- More than 10 hours

26 **How many hours do you usually sleep at night (or your main sleep period) on weekends or your non-work days?**

- Less than 6 hours
- 6 hours
- 7 hours
- 8 hours
- 9 hours
- 10 hours
- More than 10 hours

27 **On average, during the *past year,* how often have you felt sleepy during the day, no matter how much sleep you had?**

- Never
- Rarely (one day per month or less)
- Sometimes (2–4 days per month)
- Often (5–15 days per month)
- Almost always (16–30 times per month)

28 **On average, during the *past year,* how often have you ever taken sleeping pills, melatonin, or other medicine to help you sleep?**

- Never
- Rarely (once per month or less)
- Sometimes (2–4 times per month)
- Often (5–15 times per month)
- Almost always (16–30 times per month)

29 **The questions below ask how many <u>servings</u> of the following items you ate or drank during the *past year.***

During the PAST YEAR, how often did you eat or drink . . .	Never or less than once a month	1–3 times a month	1–2 times a week	3–4 times a week	5–6 times a week	1 time a day	2 times a day	3 times a day	4 times a day	5+ times a day
100% orange juice	○	○	○	○	○	○	○	○	○	○
100% grapefruit juice	○	○	○	○	○	○	○	○	○	○
Other 100% fruit juices (not counting fruit drinks)	○	○	○	○	○	○	○	○	○	○
Fruit (not counting juices)	○	○	○	○	○	○	○	○	○	○
Green salads (with or without other vegetables)	○	○	○	○	○	○	○	○	○	○
French fries or fried potatoes	○	○	○	○	○	○	○	○	○	○
Baked, boiled or mashed potatoes	○	○	○	○	○	○	○	○	○	○
Vegetables (not counting salads or potatoes)	○	○	○	○	○	○	○	○	○	○

30 **In the *past year,* about how often did you drink tea—hot or iced, caffeinated or decaffeinated (not herbal)?**

- ○ Never
- ○ 1–3 cups per month
- ○ 1–2 cups per week
- ○ 3–4 cups per week
- ○ 5–6 cups per week
- ○ 1 cup per day
- ○ 2 cups per day
- ○ 3 cups per day
- ○ 4 cups per day
- ○ 5 or more cups per day

If you drink <u>hot tea</u>, do you add milk to your hot tea?

- ○ No
- ○ Yes
- ○ Do not drink hot tea

31 **In the *past year,* about how often did you drink wine? (1 glass = 5 oz. serving)**

- ○ Never or seldom
- ○ 1–3 glasses per month
- ○ 1–2 glasses per week
- ○ 3–4 glasses per week
- ○ 5–6 glasses per week
- ○ 1 glass per day
- ○ 2 glasses per day
- ○ 3 glasses per day
- ○ 4 glasses per day
- ○ 5 or more glasses per day

32 **If you drink wine, what kind of wine do you <u>*usually*</u> drink?**

- ○ White
- ○ Red
- ○ Blush

COMMENTS

THANK YOU

PLEASE CHECK TO MAKE SURE YOU HAVE NOT ACCIDENTALLY SKIPPED ANY PAGES.

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CLUE II

STUDY NO.

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Please check the information above and correct any mistakes.

Is the birthdate indicated above correct?

○ No ➡ If no, please write correct date. ___/___/___ MONTH DAY YEAR

○ Yes, birth date is correct.

If this person is DECEASED, please provide the date of death: ___/___/___ MONTH DAY YEAR

INSTRUCTIONS

THIS FORM IS DESIGNED TO BE READ BY OPTICAL SCANNING EQUIPMENT, SO IT IS IMPORTANT THAT YOU FOLLOW THESE DIRECTIONS:

1. Use **NO. 2 PENCIL**.
2. Make no stray marks on the survey.
3. Fill the circle completely with a dark mark. Please mark this way: ●
4. Mark a circle for each question.
5. Erase completely to change a response.

EXAMPLE: **Have you been told by a doctor or other health professional that you have any of the conditions listed below?**

Mark "No" or "Yes" *and* "Year of Diagnosis" for each illness.

FOR EXAMPLE:

Someone who was diagnosed with diabetes in September 1997, with high cholesterol in 1986, but who still has normal blood pressure would fill in the circles as shown.

	No	Yes	YEAR OF DIAGNOSIS: 1989 or before	1990–1994	1995 or after
Diabetes mellitus	○	●	○	○	●
High cholesterol	○	●	●	○	○
High blood pressure	●	○	○	○	○

IF YOU HAVE ANY QUESTIONS, PLEASE FEEL FREE TO CALL OUR OFFICE AT (301) 791-3230.

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1 **How long have you lived at your current address?**

- ○ Less than 1 year
- ○ 1–2 years
- ○ 3–5 years
- ○ 6–9 years
- ○ 10–14 years
- ○ 15–19 years
- ○ 20–24 years
- ○ 25 or more years

2 **What is your marital status?**

- ○ Never married
- ○ Married
- ○ Widowed
- ○ Divorced
- ○ Separated

3 **How many grades of school, including college, have you completed?**

GRADES

0	0
1	1
2	2
	3
	4
	5
	6
	7
	8
	9

Note: It is important that you write in the grades of school completed in addition to completing the corresponding circles. This allows us to confirm that the correct circles have been filled in.

4 **How much do you weigh? (Write in number and darken circles)**

POUNDS

0	0	0
1	1	1
2	2	2
3	3	3
4	4	4
5	5	5
	6	6
	7	7
	8	8
	9	9

Note: It is important that you write in your weight in addition to completing the corresponding circles. This allows us to confirm that the correct circles have been filled in.

5 **How many hours per week do you spend either sitting or lying down while watching TV, playing video/computer games or on the internet?**

Number of hours per week

0	Less than 1 hour	1	2–3	4–6	7–10	11–20	21–30	31–40	40+
▼	▼	▼	▼	▼	▼	▼	▼	▼	▼
○	○	○	○	○	○	○	○	○	○

HEALTH HISTORY

6 **Have you been told that you have cancer?**

	No	Yes	YEAR OF DIAGNOSIS: 1989 or before	1990–1994	1995 or after
	○	○	○	○	○

➡ *If "No", go to question 9.*

7 **If you had cancer, what type(s) of cancer did you have? (Please mark below)**

TYPES OF CANCER

- ○ Bladder
- ○ Breast
- ○ Cervix
- ○ Cervix in situ
- ○ Colon or rectum
- ○ Leukemia
- ○ Lung
- ○ Lymphoma or Hodgkins
- ○ Melanoma
- ○ Oral cavity
- ○ Esophageal
- ○ Ovary
- ○ Pancreas
- ○ Prostate
- ○ Skin (basal or squamous)
- ○ Uterus or endometrium
- ○ Thyroid
- ○ Stomach
- ○ Kidney
- ○ Other (Please specify) _____

➡ **Age at diagnosis of FIRST cancer (excluding skin cancer):**

- ○ Under 20
- ○ 20–39
- ○ 40–49
- ○ 50–59
- ○ 60–69
- ○ 70 or over

8 **Where was the cancer diagnosed?**

- ○ Washington County Hospital
- ○ Other (Please specify hospital or office and city, state) _____

9 **Please mark below if your mother, father, sisters, or brothers have had the following:**

	Colorectal cancer	Prostate cancer	Breast cancer	Other Cancer	Heart attacks	Diabetes
Biological Mother	○		○	○	○	○
Biological Father	○	○	○	○	○	○
Blood-related Sisters	○		○	○	○	○
Blood-related Brothers	○	○	○	○	○	○

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10 **Have you ever been told by a doctor or other health professional that you have any of the conditions listed below? (If you don't recognize the term below, you probably haven't been diagnosed with it.) Mark either no or yes for each item. If yes, mark year of diagnosis.**

	No	Yes	YEAR OF DIAGNOSIS 1989 or before	1990–1994	1995 or after
Heart or Blood Pressure					
a. Diabetes					
b. High blood pressure (excluding during pregnancy)					
c. High cholesterol					
d. High triglycerides					
e. Heart attack					
f. Angina pectoris					
g. Stroke					
h. TIA (transient ischemic attack)					
i. Peripheral artery disease or claudication of legs (pain with walking or exercise) (not varicose veins)					
j. Arrythmias					
k. Blood clots (deep vein thrombosis)					
Bones					
l. Osteoporosis					
m. Hip fracture					
n. Herniated lumbar disk					
o. Wrist or Colles' fracture					
p. Vertebral body fracture					
Thyroid					
q. Hyperthyroid/Graves disease					
r. Hypothyroid disease					
s. Thyroid nodule					

	No	Yes	YEAR OF DIAGNOSIS 1989 or before	1990–1994	1995 or after
Arthritis/Autoimmune Disease					
t. Rheumatoid arthritis					
u. Osteoarthritis					
v. Systemic lupus					
w. Gout					
Gastrointestinal					
x. Colon or rectal polyp (benign)					
y. Gallbladder disease/gallstones					
z. Gastric or duodenal ulcer					
aa. Hiatal hernia					
bb. Chronic indigestion					
cc. Diverticulitis/diverticulosis					
dd. Ulcerative colitis/Crohn's disease					
ee. Barrett's esophagus					
Kidney/Bladder					
ff. End stage renal disease					
gg. Kidney stones					
Lung					
hh. Asthma					
ii. Emphysema or chronic bronchitis					
Neurologic Conditions					
jj. Migraine headaches					
kk. Multiple Sclerosis					
ll. Parkinson's disease					

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	No	Yes	YEAR OF DIAGNOSIS 1989 or before	1990–1994	1995 or after
Teeth					
mm. Peridontal disease (gum disease)					
nn. Tooth loss					
Eyes					
oo. Macular degeneration of the retina					
pp. Cataract					
Breast/gynecologic conditions					
qq. Fibrocystic disease of the breast or other benign breast disease					
rr. Endometriosis					
ss. Uterine fibroids					
tt. Infertility					
Prostate					
uu. Enlarged prostate (Benign prostatic hyperplasia)					
Other major medical condition					
vv. Other (Specify condition) _____					

11 In the past two years, have you had any of the following tests of your colon (large intestine)? (Please mark all that apply.)

○ None → ***If "None", go to question 13.*** ○ Fecal occult blood test ○ Sigmoidoscopy ○ Colonoscopy

12 Why did you have the colonoscopy or sigmoidoscopy? (Mark all that apply.)

○ Saw blood in stool
○ Abdominal pain
○ Family history of colon or rectal cancer
○ Diarrhea or constipation
○ Positive test for blood in stool
○ Routine or follow-up screening (no symptoms)
○ Abnormal barium enema
○ Previous polyp
○ Previous colon or rectal cancer

13 Have you had your cholesterol level checked within the past 2 years?

○ No ○ Yes → (What was your level?_____) ***Was it high?*** ○ No ○ Yes

14 During the past two years on average, how often have you taken the following medications? How many tablets have you taken on average? (Please answer each item below.)

Medications	Never or less than one a month	1–3 per month	1–3 per week	4–6 per week	1 per day	2 per day	3 per day	4 or more per day
Aspirin—Baby or low-dose (162 mg or less)	○	○	○	○	○	○	○	○
Aspirin—Regular or extra-strength aspirin—For example: Bufferin, Anacin, Bayer, Excedrin, Ecotrin, etc.	○	○	○	○	○	○	○	○
Acetaminophen—For example: Tylenol, Phenaphen, etc.	○	○	○	○	○	○	○	○
Anti-inflammatory analgesics (Cox-2 inhibitors)—Celebrex (celecoxib), Vioxx (rofecoxib)	○	○	○	○	○	○	○	○
Other Anti-inflammatory analgesics (other than aspirin)—For example: Ibuprofen, Motrin, Advil, Naprosyn, Anaprox, Aleve, Voltaren, Feldene, Toradol, Indocin, etc.	○	○	○	○	○	○	○	○

15 Have you ever taken any of the following medications: Mark either no or yes for each item. If yes, write year.

FOR HEART OR BLOOD PRESSURE: Medications	No	Yes	If "Yes" → Year started	No. of years taken
Calcium Channel Blocker—For example: Procardia, Cardizem, Norvase, Calan, Adalat, Sudar, Yerapamil, Amlodipine, etc.	○	○	____	____
Beta Blocker—For example: Lopressor, Tenormin, Inderal, Atenolol, Metroprolol, etc.	○	○	____	____
ACE Inhibitor—For example: Vasotec, Zestril, Capoten, Prinivil, Lotensin, Accupril, Monopril, Captopril, etc.	○	○	____	____
Diuretic—For example: Lasix, Lozol, Triamterene, HCTZ, Turosemide, Thiazides, etc.	○	○	____	____
Other (Specify name) _____	○	○	____	____

TO REDUCE CHOLESTEROL: Medications	No	Yes	If "Yes" → Year started	No. of years taken
Pravachol (Pravastatin)	○	○	____	____
Lipitor (Atorvastatin)	○	○	____	____
Mevacor (Lovastatin)	○	○	____	____
Lescol (Fluvstatin)	○	○	____	____
Zocor (Simvastatin)	○	○	____	____
Other (Specify name) _____	○	○	____	____

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FOR OSTEOPOROSIS: Mark either no or yes for each item. If yes, write year.

Medications	No	Yes	If "Yes"	Year started	No. of years taken
Evista (Raloxifene)	○	○		____	____
Miacalcin (Calcitonin)	○	○		____	____
Fosamax (Alendronate)	○	○		____	____
Didronel	○	○		____	____
Tamoxifen	○	○		____	____
Calcium	○	○		____	____
Vitamin D	○	○		____	____
Other (Specify name) _____	○	○		____	____

HORMONES

Medications	No	Yes	If "Yes"	Year started	No. of years taken
Testosterone (Androderm, Android)	○	○		____	____
Estrogen alone (e.g., Premarin, Estrace, Estradiol)	○	○		____	____
Estrogen and progesterone (e.g., Premarin and Provera, Prempro)	○	○		____	____
Tamoxifen (Nolvadex)	○	○		____	____
Raloxifene (Evista)	○	○		____	____
Other (Specify name of hormone) _____	○	○		____	____

16 Have you ever had any of the following procedures?

Procedure	What year did you have this procedure?	Where was it done?
Coronary bypass or angioplasty		
○ No ○ Yes	_____ Year	_____
Carotid surgery (endarterectomy)		
○ No ○ Yes	_____ Year	_____
Cardiac catherization (angiogram)		
○ No ○ Yes	_____ Year	_____
Cerebral or carotid angiogram		
○ No ○ Yes	_____ Year	_____
Dialysis (for kidney disease)		
○ No ○ Yes	_____ Year	_____

17 In general, would you say your health is:

○ Excellent ○ Very good ○ Good ○ Fair ○ Poor

18 Does pain usually interfere with your normal work (including both work outside the home and housework)?

○ Not at all ○ A little bit ○ Moderately ○ Quite a bit ○ Extremely

19 During the past year, how much of the time has your physical health interfered with your social activities (like visiting with friends, relatives, etc.)?

○ None of the time ○ A little of the time ○ Some of the time ○ Most of the time ○ All of the time

20 What types of health care coverage or insurance do you currently use to pay for <u>most</u> of your medical care? (Mark only the most important)

○ Medicare plus other insurance

○ Medicare plus Medicaid

○ Medicare only

○ A plan provided by your employer

○ A plan provided by someone else's employer

○ A plan that you or someone else buys on your own

○ Medicaid, Medical Assistance, Health Choice, or Maryland Children's Health Program

○ The military, CHAMPUS, or the VA, Tricare

○ Some other source (Specify) _____

○ Don't have health care coverage or health insurance

Is any of your coverage/insurance through an HMO, MCO, POS? ○ No ○ Yes

21 Are there particular clinics, health centers, doctor's offices or other places that you usually go to if you are sick or need advice about your health?

○ No ➡ ***If "No", Go to Question 23.*** ○ Yes ○ Don't know/Not sure

22 Is this place a clinic, a hospital, a doctor's office, or some other place? (Mark all that apply.)

○ Physician's office

○ Community Free Clinic

○ Potomac Street Clinic or Walnut Street Community Health Clinic

○ Health Department

○ Hospital Express Care

○ Hospital Emergency Room

○ Martinsburg VA Center

○ Hagerstown VA Center (Western Maryland Hospital)

○ Other (specify) _____

○ Don't know/Not sure

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TOBACCO USE

23A Have you ever used any tobacco products?

○ No ➡ ***(If no, go to Question 26.)*** ○ Yes

23B Indicate your use of these tobacco products. Mark all that apply.

	Cigarettes	Cigars	Pipe	Snuff	Chewing Tobacco
Previous use, but quit	○	○	○	○	○
Current use	○	○	○	○	○

24 If you have ever smoked cigarettes, how many do you or did you smoke each day?

○ Never smoked cigarettes ➡ ***(Go to Question 26.)*** ○ 1–4 ○ 15–24 ○ 35 or more

○ Less than 1 ○ 5–14 ○ 25–34

25 Have you ever tried to quit smoking using any of the following methods? Mark either no or yes for each item. If yes, mark how long you used it.

Method	No	Yes	If "Yes" ➡ <1 month	1–6 months	7–12 months	>12 months
			If yes, for how long did you use it?			
Individual/group counseling	○	○	○	○	○	○
Nicotine gum (Nicorette)	○	○	○	○	○	○
Nicotine patch (Habitrol, Nicoderm CQ, Nicotrol, ProStep)	○	○	○	○	○	○
Nicotine nasal spray (Nicotrol NS)	○	○	○	○	○	○
Nicotine inhaler (Nicotrol)	○	○	○	○	○	○
Non-nicotine medication (e.g., Zyban, Wellbutrin)	○	○	○	○	○	○
Hypnosis	○	○	○	○	○	○
Acupuncture	○	○	○	○	○	○
Quitting on your own – "Cold turkey"	○	○				

If you marked "Yes" to TWO OR MORE choices listed above, which was the MOST successful in helping you to quit smoking?

○ Individual/group counseling by itself

○ Individual/group counseling along with a nicotine product

○ Individual/group counseling along with a non-nicotine medication

○ Nicotine gum

○ Nicotine patch

○ Nicotine nasal spray

○ Nicotine inhaler

○ Non-nicotine medication

○ Hypnosis

○ Acupuncture

○ Quitting on your own – "Cold turkey"

VITAMINS

26 Do you currently take a multi-vitamin? (Please report other individual vitamins in Question 27.)

○ No ○ Yes →

a. How many do you take per week?

○ 2 or less ○ 3–5 ○ 6–9 ○ 10 or more

b. What specific brand do you usually use? _____

27 Do you take the following separate supplements? DO NOT REPORT CONTENTS OF MULTI-VITAMINS MENTIONED ABOVE. Mark either no or yes for each item. If yes, mark dose.

			Dose per day					
A. Vitamin A	○ No	○ Yes →		○ less than 8,000 IU	○ 8,000 to 12,000 IU	○ 13,000 to 22,000 IU	○ 23,000 IU or more	○ Don't know
B. Beta-carotene	○ No	○ Yes →		○ less than 8,000 IU	○ 8,000 to 12,000 IU	○ 13,000 to 22,000 IU	○ 23,000 IU or more	○ Don't know
C. Vitamin C	○ No	○ Yes →		○ less than 400 mg	○ 400 to 700 mg	○ 750 to 1250 mg	○ 1300 mg or more	○ Don't know
D. B-Complex	○ No	○ Yes						
E. Vitamin B_6	○ No	○ Yes →		○ less than 10 mg	○ 10 to 39 mg	○ 40 to 79 mg	○ 80 mg or more	○ Don't know
F. Folic Acid	○ No	○ Yes →		○ less than 400 mcg	○ 400 to 799 mcg	○ 800 to 1199 mcg	○ 1200 mcg or more	○ Don't know
G. Vitamin B_{12}	○ No	○ Yes →		○ less than 100 mcg	○ 100 to 249 mcg	○ 250 to 499 mcg	○ 500 mcg or more	○ Don't know
H. Vitamin E	○ No	○ Yes →		○ less than 100 IU	○ 100 to 250 IU	○ 300 to 500 IU	○ 600 IU or more	○ Don't know
I. Calcium (Include calcium in Tums, etc.) (1 Tum = 200 mg elemental calcium)	○ No	○ Yes →	Dose per day (elemental calcium)	○ less than 400 mg	○ 400 to 900 mg	○ 901 to 1300 mg	○ 1301 mg or more	○ Don't know
J. Selenium	○ No	○ Yes →		○ less than 80 mcg	○ 80 to 130 mcg	○ 140 to 250 mcg	○ 260 mcg or more	○ Don't know
K. Niacin	○ No	○ Yes →		○ less than 50 mg	○ 50 to 300 mg	○ 400 to 800 mg	○ 900 mg or more	○ Don't know
L. Zinc	○ No	○ Yes →		○ less than 25 mg	○ 25 to 74 mg	○ 75 to 100 mg	○ 101 mg or more	○ Don't know

M. Are there other supplements that you take on a regular basis?

Iron	Magnesium	Soy supplements	Bee pollen
SAMe (S-adenosylmethionine)	Melatonin	Fish oil	Shark cartilage
Coenzyme Q10	DHEA	Ginseng	Cod liver oil
Gingko biloba	Saw Palmetto	Flaxseed or oil	Vitamin D
Antioxidant supplements	Echinacea	Metamucil/Citrucil	Other (please specify)
St. Johns wort	Chromium	Blue Green algae	_____
Potassium	Lecithin	Garlic supplements	_____

DIETARY QUESTIONS

28 During the past year how often did you eat or drink the following:	Never or less than once a month	1–3 times a month	1–2 times a week	3–4 times a week	5–6 times a week	1 time a day	2+ times a day
100% orange juice							
100% grapefruit juice							
Other 100% fruit juices (not counting fruit drinks)							
Dried fruits, like raisins, apricots, dates							
Any other fruit (fresh, canned, or frozen) but not counting juices							
100% vegetable juice, like tomato or V8							
Green salad, like lettuce or spinach							
Spaghetti sauce or tomato sauce							
Vegetable soup or stew with vegetables							
Beans, like baked beans, pinto or kidney beans, lentils (cooked from dried beans or canned), but not including green beans							
French fries or fried potatoes							
Baked, boiled or mashed potatoes							
Any other vegetables, like green beans, peas, corn, broccoli, tomatoes (fresh, frozen, or canned)							

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29 **During the past year, how often did you drink a glass or a cup of . . .**

	Never or less than once a month	1–3 times a month	1 time a week	2–3 times a week	4–6 times a week	2–3 times a day	4–5 times a day	6+ times a day
Red wine	○	○	○	○	○	○	○	○
White wine	○	○	○	○	○	○	○	○
Beer (1 glass, can, or bottle)	○	○	○	○	○	○	○	○
Liquor (1 ounce shot)	○	○	○	○	○	○	○	○
Coffee	○	○	○	○	○	○	○	○
Green tea (hot or iced)	○	○	○	○	○	○	○	○
Black tea (hot or iced)	○	○	○	○	○	○	○	○
Water	○	○	○	○	○	○	○	○
Milk	○	○	○	○	○	○	○	○
Soft drinks	○	○	○	○	○	○	○	○
Other (Specify) _____	○	○	○	○	○	○	○	○

30 **When you drink coffee, tea, or soda, about how often do you drink them <u>without</u> caffeine?**

○ Never ○ Half the time ○ Always

○ Rarely ○ Usually

MEN'S HEALTH

The following questions are important for our learning about the causes of prostate cancer, and other prostate diseases.

31 **In the past two years, has a physician checked your prostate by rectal examination?**

○ No ○ Yes ○ Don't know

32 **During the past two years, have you had a blood test (PSA) to see if you had prostate cancer?**

○ No **(If no, go to Question 35)** ○ Yes **(Continue with Question 33)**

33 Was the PSA blood test abnormal?

○ No (If no, go to Question 35) ○ Yes (Continue with Question 34)

34 Abnormal PSA followed up by: (Mark all that apply.)

○ Not followed up ○ Biopsy ○ Hormone treatment

○ Repeat test ○ Surgical operation

○ Ultrasound ○ Radiation

35 On average, how many times per night do you usually get up to urinate?

○ 0 ○ 1 ○ 2 ○ 3 ○ 4 ○ 5+

36 During the past month, please indicate how frequently you had these urinary symptoms:

	% of time experienced symptoms					
	Never	10%	25%	50%	75%	100%
Sensation of incomplete bladder emptying	○	○	○	○	○	○
Having to urinate again after less than 2 hours	○	○	○	○	○	○
Stopping and starting several times during urination	○	○	○	○	○	○
Found it difficult to postpone urinating	○	○	○	○	○	○
Weak urinary stream	○	○	○	○	○	○
Had to push or strain to begin urination	○	○	○	○	○	○

37 In the past two years, have you regularly taken medication to treat an enlarged prostate or to treat the urinary symptoms of benign prostatic hyperplasia (BPH)? Mark either no or yes for each item.

	No	Yes
Finasteride (e.g., Proscar)	○	○
Doxazosin (Cardura)	○	○
Terazosin (Hytrin)	○	○
Tamsulosin (Flomax)	○	○
Saw Palmetto	○	○
Other (Specify name) _____	○	○

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217374-4/5

38 **Have you ever been diagnosed with prostatitis or a prostate infection?**

○ No ○ Yes, once ○ Yes, more than once

39 **Please mark your hair pattern at age 45 or your hair pattern now if you are under age 45.**

○ None or minimal

40 **Have you ever had a vasectomy (Male Sterilization)?**

○ No ○ Yes

What year did you have the surgery?

_____ Year

Where was the surgery done?

○ Washington County Hospital

○ Other, specify doctor's office or hospital, city, state:

_____

_____

41 **Have you ever had PROSTATE SURGERY?**

○ No ○ Yes

If yes, what type of surgery?

Transurethral Resection (TURP)

○ No ○ Yes

What year did you have the surgery?

_____ Year

Where was the surgery done?

○ Washington County Hospital

○ Other, specify doctor's office or hospital, city, state:

_____

_____

Biopsy

○ No ○ Yes

_____ Year

○ Washington County Hospital

○ Other, specify doctor's office or hospital, city, state:

_____

_____

Prostatectomy (removal of the prostate gland)

○ No ○ Yes

_____ Year

○ Washington County Hospital

○ Other, specify doctor's office or hospital, city, state:

_____

_____

WOMEN'S HEALTH

42 During the past two years, have you had a:

	No	Yes
Pelvic exam	○	○
Pap smear test	○	○
Breast exam by a health professional	○	○
Mammogram	○	○

43 Have your MENSTRUAL PERIODS stopped permanently?

- ○ No, still menstruating. ➡ ***Go to question 46.***
- ○ Had menopause, but now have periods due to hormone replacement therapy.
- ○ Yes, menstrual periods stopped.

44 How old were you when your natural MENSTRUAL PERIODS stopped?

Age periods stopped (Write in number and darken circles)

Age		
0	○	○
1	○	○
2	○	○
3	○	○
4	○	○
5	○	○
6	○	○
7		○
8		○
9		○

45 Why did your natural MENSTRUAL PERIODS stop?

- ○ Surgical menopause (hysterectomy or removal of ovaries and/or uterus)
- ○ Natural menopause (Change of Life)
- ○ Radiation/Chemotherapy
- ○ Other, specify _____

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46 Have you ever had any of the following procedures?

Procedure	What year(s) did you have this procedure?	Where was the procedure done?
BREAST BIOPSY WITH A NEEDLE OR SURGERY (Do not count removing fluid with a needle.) ○ No ○ Yes **IF YES,** number of biopsies ○ One ○ Two ○ Three or more Did any biopsy show breast cancer? ○ No ○ Yes	_____ Year _____ _____	○ Washington County Hospital ○ Other, specify doctor's office or hospital, city, state: _____ _____ _____
HYSTERECTOMY (removal of uterus) ○ No ○ Yes	_____ Year	○ Washington County Hospital ○ Other, specify doctor's office or hospital, city, state: _____ _____ _____
OOPHORECTOMY (removal of ovaries) ○ No ○ Yes ○ One ovary ○ Both ovaries	_____ Year	○ Washington County Hospital ○ Other, specify doctor's office or hospital, city, state: _____ _____ _____
TUBAL LIGATION (tubes tied) ○ No ○ Yes	_____ Year	○ Washington County Hospital ○ Other, specify doctor's office or hospital, city, state: _____ _____ _____

COMMENTS

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Letters

Temperature Variations in Chest-type Mechanical Freezers

To the Editor:

A recent report described temperature variations within upright mechanical freezers set at −80 °C *(1)*. The range of internal temperatures was great, with the warmest specimen being at −43.5 °C and the coldest at −90 °C. These findings suggested that it might be useful to study the temperature variation within horizontal chest-type freezers.

In 1974, blood samples were obtained from 25 802 adult volunteers in Washington County, Maryland. In 1989, an additional 32 320 samples were obtained in a similar project. The separated serum samples from each project were stored in mechanical chest-type freezers set to hold an interior temperature of −73.3 °C. For subsequent studies, sera from newly diagnosed cancer cases and from healthy controls have been assayed for a variety of micronutrients, hormones, or antibodies.

In this study, serum samples were stored in 5-mL plastic Nunc tubes (Nunc), 100 in a cardboard box with the boxes stacked in racks in two sizes of freezers. Five freezers (So-Low Environmental Equipment Company) were used in the study. Two were 27-cubic foot freezers (model no. SE 27-100) holding racks that are seven boxes high with four racks front to back and 11 across, for a total capacity of 30 800 samples. Three freezers (model no. PR100-7) had a capacity of 7 cubic feet. Two held 2.5 racks front-to-back and eight across, for a total capacity of 8000 specimens. The third small freezer contained approximately one-half of its total capacity.

For temperature measurements, 22-gauge copper-constantan type T thermocouples were used, each threaded through the cap of a 5-mL Nunc tube of the same type as those containing serum samples, but with the thermocouple tip immersed in 3 mL of tap water. The tubes containing thermocouples were placed in the outer row of tubes in a box.

Temperatures were measured in degrees Fahrenheit, using an Omega model HH-25TF thermometer with a crystal display (Omega Engineering). The calibration of the Omega thermocouple indicator was verified by a Thermo Electric Micromite II thermocouple calibrator, which was traceable to NIST. Although all temperatures in this study were recorded in degrees Fahrenheit, we are reporting in degrees Celsius to simplify comparisons with other studies.

Temperatures were measured in sets of eight tubes per freezer. The tubes were placed in boxes at the top and bottom of each freezer, close to each of the four corners. A minimum of 24 h was allowed for stabilization before temperatures were recorded.

To determine the extent to which the thermocouple leads might cause heat leakage into the freezers, the ends at which leads were inserted were alternated for the freezers tested, and top temperatures were compared between the thermocouple end and the other sealed end. The mean temperature difference between the two ends was only 0.1 °C.

Room temperatures during the study period were 20–25 °C. We could not detect any effect of this limited ambient temperature range on the study results.

The mean temperature differences between top and bottom varied by freezer capacity. For the 27-cubic foot freezers, the temperature differential was 15.9 °C with a difference of only 1.1 °C between the two freezers. For the two fully loaded small freezers, the mean top-to-bottom difference was 10.1 °C, and the difference between the two freezers was 2.4 °C. The small freezer filled to only one-half of its capacity had a top-to-bottom difference of 10.6 °C.

This study indicates that chest-type freezers provide greater uniformity of temperature than the upright front-door type. It also suggests that it might be prudent to match cases and controls based on the part of the freezer in which the specimens had been stored. Top-to-bottom temperature differentials of 10–15 °C are not trivial. Although they may have no deleterious effects on study analytes after short-term storage, this may not hold true when storage times are measured in years or decades.

A limitation of this study is that it involved only a few freezers from a single manufacturer and only a limited range of ambient temperatures. However, we can think of no reason that temperature variations within other makes of chest-type freezers should differ appreciably from those we observed. Temperature variation within freezers is likely to be a pervasive problem that warrants further investigation.

This research was supported in part by DAMD Department of the Army Grant 17-94-J-4265, and by Research Career Award HL 21670 from the National Heart, Lung, and Blood Institute (to G.W.C.). We thank Douglas H. Smith of Mack Trucks, Inc. for technical assistance.

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Knud J. Helsing‡
Sandra C. Hoffman
George W. Comstock*

Training Center
for Public Health Research
Johns Hopkins University
School of Hygiene and Public Health
Box 2067
Hagerstown, MD 21742-2067

‡Deceased.
*Author for correspondence. Fax 301-797-3669; e-mail gcomstock@Mindspring.com.

Effects of Repeated Freeze-Thaw Cycles on Concentrations of Cholesterol, Micronutrients, and Hormones in Human Plasma and Serum, *George W. Comstock,[1*] Alyce E. Burke,[1] Edward P. Norkus,[2] Gary B. Gordon,[3] Sandra C. Hoffman,[1] and Kathy J. Helzlsouer[1]* ([1] Department of Epidemiology, Johns Hopkins School of Public Health, Hagerstown, MD 21742; [2] Department of Medical Research, Our Lady of Mercy Medical Center, Bronx, NY 10470; [3] Searle, Skokie, IL 60077; * address correspondence to this author at: Johns Hopkins Training Center for Public Health Research, 1302 Pennsylvania Ave., Hagerstown, MD 21742-3197; fax 301-797-3669, e-mail gcomstock@mindspring.com)

The effects of repeated freeze-thaw cycles on concentrations of various analytes in plasma or serum were of little interest until the growth of plasma and serum banks during the latter part of the 20th century. By 1996, the number of such banks used primarily for cancer research had grown to 115 *(1)*. Many also exist for other purposes, such as the WHO Serum Reference Banks *(2)* and banks associated with cardiovascular studies such as the Multiple Risk Factor Intervention Trial (MRFIT) and the Atherosclerosis Risk in Communities (ARIC) Study *(3, 4)*. Although the need for repeated freezing and thawing of samples can be minimized by storing banked specimens in several small containers *(5)*, it often is necessary to use plasma or serum that has already undergone one or more freeze-thaw cycles. When this occurs, reviewers of re-

search protocols or manuscripts may question the validity of data obtained from these specimens.

The scientific literature provides few answers to such questions. Medline contains no appropriate key word or phrase for searching; comments on the effects of freeze-thaw cycles often are limited to a few sentences in publications that focus on stability during long-term storage or on assay methodology. The present study was designed to add information to this sparse literature.

In 1991, 10 healthy adult volunteers, 5 men and 5 women, each donated 120 mL of blood. The purpose of the study was explained to each person along with the risks and lack of individual benefit. Approval of the study was granted by the Committee on Human Volunteers of the Johns Hopkins School of Hygiene and Public Health before study initiation.

Blood was collected into three 20-mL Vacutainer Tubes containing 286 USP units of sodium heparin (cat. no. 6406; Becton Dickinson) to obtain plasma, and into four 15-mL plain Vacutainer Tubes to obtain serum. The three plasma samples and four serum samples were pooled for each donor. Each of these 10 plasma and serum donor pools was divided into 7 primary aliquots, 2 for baseline assays after one freeze-thaw cycle, and 1 each for the assays after 2, 3, 4, 6, and 10 freeze-thaw cycles. From each primary plasma aliquot, five secondary aliquots were prepared for assays of ascorbic acid, cholesterol, dehydroepiandrosterone and dehydroepiandrosterone sulfate, other hormones, and micronutrients. Each primary serum aliquot was similarly divided except that no ascorbic acid assays were done on serum. Plasma and serum samples were kept in sterile flasks set in ice-water except during the actual fluid transfers. For the ascorbic acid assays, 0.5 mL of freshly prepared 100 g/L metaphosphoric acid was added to 0.5 mL of plasma before freezing; the mixture was then immediately frozen at −70 °C. All aliquoting and thawing were done under dim yellow light.

Secondary aliquots for freeze-thaw cycle 2 were removed from the freezer, thawed once in cold water, allowed to stand at room temperature for 30 min, and refrozen at −70 °C. These samples were not thawed again until the time of assay, thereby completing the second freeze-thaw cycle. Samples for freeze-thaw cycle 3 were thawed as above, refrozen at −70 °C, thawed as above again, and then kept frozen until thawed for assay. This system of thawing and refreezing was repeated so that freeze-thaw cycle 4 had its fourth freeze-thaw cycle completed at the time of assay. These procedures were continued for freeze-thaw cycles 6 and 10.

All specimens remained frozen in insulated containers with dry ice during shipment to the assay laboratories. All specimens were assayed in the random order assigned before shipment. This procedure ensured that the order of assaying was random with respect to both the donor of the specimens and to the freeze-thaw cycle.

Ascorbic acid assays were performed with 2,4-dinitrophenylhydrazine as chromogen *(6)*. Other micronutrients (retinol, total carotenoids, α-carotene, β-carotene, cryptoxanthin, lycopene, lutein, α-tocopherol, and γ-tocopherol) were assayed by reversed-phase HPLC *(7)*. Cholesterol concentrations were determined enzymatically *(8)*. Assays for dehydroepiandrosterone and its sulfate were performed by RIA (Wien Laboratories, Succasunna, NJ) *(9)*. The procedure was that suggested by the manufacturer except that dehydroepiandrosterone was extracted with a 1:1 mixture (by volume) of dichloromethane and hexane.

For males, the hormones for assay were estrone, estradiol, testosterone, and sex hormone-binding globulin (SHBG); for females, androstenedione, follicle-stimulating hormone, luteinizing hormone, progesterone, and SHBG were determined. In male serum, estrone and estradiol were measured by RIA after extraction and Celite chromatography *(10)*. Testosterone was measured by RIA using a method from DPC. SHBG was measured using an immunoradiometric method from Orion. In female serum, androsterone and progesterone were measured by RIA using methods from ICN.

The results for duplicate aliquots that had undergone only one freeze-thaw cycle were used to estimate imprecision (as CVs) for the individual assays. The mean value of the two concentrations for each analyte was used as the cycle 1 value. For each analyte, the linear regression equation ($y = a + bx$) for mean analyte concentrations (y) on the number (x) of freeze-thaw cycles was calculated. The average amount of change per cycle (b) was divided by the estimated value before any freezing (a), and the result was expressed as a percentage of the calculated prefreezing value.

The imprecision (CV) for plasma and serum was similar. For cholesterol and the micronutrients, the median CV was 7.4%, with a range of 1.2% (serum cholesterol) to 19% (plasma α-carotene). For hormones, the variability was greater. The median CV was 14% and the range was 2.5% (androstenedione in serum) to 58% (progesterone in serum). For seven analytes, CVs were >15%: progesterone in plasma and serum (41% and 58%, respectively); estradiol in plasma and serum (17% and 39%); estrone in plasma and serum (27% and 22%); and dehydroepiandrosterone in plasma (17%).

The mean change associated with each freeze-thaw cycle (and its accompanying 30-min exposure to room temperature) was <4% of the estimated prefreeze concentration for all analytes and <2% for nearly all of them (Table 1). Five analytes had changes per cycle of 2–4%. All of these five were hormones: estrone in plasma and serum; estradiol in serum; and SHBG and dehydroepiandrosterone sulfate in plasma. In general, there was a slight tendency for concentrations to decrease with each successive freeze-thaw cycle and for these changes to be least for the first three cycles. Analyte concentrations in serum were somewhat less likely to be affected by freezing and thawing than concentrations in plasma.

In this study, three cycles of freezing and thawing had almost no effect on concentrations of cholesterol, micronutrients, and most of the hormones investigated (data not shown). For estrone, estradiol, testosterone, and SHBG, there was appreciable variation within the first

three cycles, but the degree of variability was considerably less than the respective CVs. Although there were greater losses after 6 or 10 cycles for a few other analytes, these were too small to have a meaningful effect on results.

With one exception, other studies agree with these findings. With respect to cholesterol, serum specimens from 10 baboons were subjected to 10 freeze-thaw cycles *(11)*. The results indicated "a stable serum cholesterol during repeated freezing-thawing".

In a report on assay methodology, Driskell et al. *(12)* stated that "Vitamins A and E in serum were found to be stable to freezing and thawing (seventeen freezing and thawing cycles over a period of five weeks)". Brioch et al. *(13)* concluded that eight freezing and thawing cycles of hypercarotenemic serum made "no significant differences in the levels of carotenoids or retinyl palmitate". In a study on the stability of vitamin E, Gunter et al. *(14)* reported that two samples stored at −70 °C for 3 months had losses of 13% and 21% after the fifth cycle. Regression analysis of data reported by Nierenberg *(15)* showed that after seven freeze-thaw cycles of a single specimen, plasma concentrations of β-carotene decreased by 0.3% of the estimated baseline value per cycle. A more comprehensive study by Hsing et al. *(16)* used 15 aliquots of pooled sera. After four freeze-thaw cycles, no changes in the concentrations of retinol, total carotenoids, β-carotene, lycopene, or total tocopherols were >0.3% of the estimated prefreezing value.

Hsing et al. *(16)* also looked at changes in serum concentrations of several hormones after three freeze-thaw cycles of five pooled specimens. The estimated change per cycle was 0.7% for testosterone, 1.4% for luteinizing hormone, and 3% for follicle-stimulating hormone. Wickings and Nieschlag *(17)* assayed four aliquots of plasma after 33 freeze-thaw cycles. They concluded that "Repeated freezing and thawing of plasma samples does not affect the plasma concentrations of T (testosterone) and Adione (androstenedione)".

On the basis of previous reports and the results of this study, we believe that repeated freezing to −70 °C and thawing has no meaningful effects on the plasma and serum concentrations of a considerable number of micronutrients and hormones.

Table 1. Mean change in concentrations of selected analytes per freeze-thaw cycle expressed as a percentage of the estimated initial value.

	Change, %			
	Plasma		Serum	
Analyte	Mean	SD	Mean	SD
Cholesterol	−0.38	0.23	−0.01	0.11
Micronutrients				
Retinol	−0.16	0.35	−0.32	0.17
Total carotenoids	−1.34	0.37	−0.28	0.23
α-Carotene	−0.63	0.44	0.12	0.50
β-Carotene	−0.84	0.64	0.15	0.63
Cryptoxanthin	−0.97	0.53	−0.48	0.47
Lutein	−0.75	0.49	−0.70	0.46
Lycopene	−1.54	1.08	−0.13	0.48
Ascorbic acid	0.14	0.12		
α-Tocopherol	−1.36	0.41	−0.44	0.09
γ-Tocopherol	−1.09	0.22	−0.47	0.27
Hormones, female				
Androstenedione	0.34	1.49	−1.09	1.12
FSH[a]	−0.70	0.50	−0.12	0.59
Luteinizing hormone	−1.60	0.87	−0.12	0.34
Progesterone	0.09	2.37	−0.31	5.93
SHBG	0.03	1.28	−0.67	2.37
Hormones, male				
Estrone	−2.21	1.04	−2.23	0.56
Estradiol	−0.33	4.45	−2.45	2.74
Testosterone	1.77	1.62	1.01	2.91
SHBG	2.73	2.65	−1.01	2.10
Hormones, both sexes				
DHEA	−0.19	1.94	−0.14	0.51
DHEAS	3.26	1.36	0.48	1.01

[a] FSH, follicle-stimulating hormone; DHEA, dehydroepiandrosterone; DHEAS, dehydrepiandrosterone sulfate.

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The Repeatability of Serum Carotenoid, Retinoid, and Tocopherol Concentrations in Specimens of Blood Collected 15 Years Apart[1]

George W. Comstock,[2] Alyce E. Burke, Sandra C. Hoffman, Edward P. Norkus, Myron Gross, and Kathy J. Helzlsouer

Department of Epidemiology, Johns Hopkins School of Hygiene and Public Health, Baltimore, Maryland 21205-2179 [G. W. C., A. E. B., S. C. H., K. J. H.]; Biomedical Research, Our Lady of Mercy Medical Center, Bronx, New York 10466-2697 [E. P. N.]; and Division of Epidemiology, School of Public Health, University of Minnesota, Minneapolis, Minnesota 55454 [M. G.]

Abstract

Community-wide programs to collect blood for a research serum bank were carried out in Washington County, Maryland in 1974 and 1989. Of the 8395 persons who participated in both programs, 64 were controls in a nested case-control study of the association of antioxidant micronutrients with subsequent breast cancer, and 30 and 166 were controls in similar studies of lung and prostate cancer. Assay results for five carotenoids, two retinoids, and two tocopherols in samples of blood collected 15 years apart were thus available for comparisons of micronutrient concentrations. The mean Spearman rank order correlation coefficient for all comparisons was 0.44, with two coefficients greater than 0.60 and two less than 0.30. Blood pressure readings at the two blood collections had a mean rank order correlation coefficient of 0.46. Because blood pressure readings in 1974 were shown to be significantly predictive of atherosclerosis 15–18 years later, the present results suggest that ranked concentrations of antioxidant micronutrients from a single sample are sufficiently representative to be used as predictors of subsequent concentrations and are thus suitable for assessment as risk factors for subsequent illnesses.

Introduction

In an extensive review of the known and potential causes of cancer, Doll and Peto (1) included the possibility that cellular damage by oxidative free radicals might be prevented by dietary antioxidants delivered to the cells by the blood and that β-carotene was a reasonable candidate for such a protective substance. This idea became the primary stimulus for numerous subsequent studies of the association of cancer with dietary and serum[3] concentrations of a variety of antioxidants, notably the carotenoids, vitamin E, vitamin C, and selenium (a marker for glutathione peroxidase).

Most of these studies have relied on histories of dietary intake or biochemical assays of serum. Dietary histories have the advantage of indicating average consumption over relatively long periods of time, but their validity suffers from the frailties of human memory and the uncertain pertinence of intake to the micronutrient concentrations in the fluids to which cells are exposed. Serum concentrations are much more likely to reflect cellular exposures, but they suffer from the fact that they are often measured at only one time and may not be representative of usual concentrations.

There are relatively few reports of intraindividual variability of serum levels of micronutrient antioxidants such as carotenoids, retinol, or tocopherols. Most are based on small numbers of subjects and have relatively short intervals between assays. All are concerned with absolute concentrations. Their findings are summarized in Table 1.

Among studies for which coefficients of variation were reported or could be calculated, these indices were 0.10 or less for shorter periods [1 day or one menstrual cycle (2, 3, 6, 7)] but ranged from 0.11–0.47 when specimens were taken a year or more apart (3, 10, 11). Correlation coefficients ranged from 0.68–0.95 for comparisons over short periods but fell to a range of 0.22–0.81 for longer intervals (8, 9, 11).

Whereas it would be ideal if individual serum concentrations remained essentially the same for long periods of time, this is unlikely. Variability can arise from changes in dietary intake, metabolic changes associated with age, and diurnal and seasonal variations. In addition, if recent and past specimens are assayed at the same time, changes due to long-term storage are possible. Even if assays are performed at the time of blood collection, it is unlikely that all assay conditions will remain constant if years separate the repeat assays. However, the significance of serum micronutrients as biomarkers for future disease can still be estimated if persons who have high concentrations at one time tend to have relatively high concentrations at a later time, and if persons with low concentrations also tend to rank low in the future. It is possible that rank order correlations will be high enough for rank, grouped or individual, to be used in analyses, even if other measures of agreement are poor. Unfortunately, only in the study among Belize children could a rank order correlation be calculated: it was 0.87 for retinol (5).

Three nested case-control studies of breast, lung, and prostate cancer in Washington County, Maryland performed repeated assays of carotenoids, retinoids, and tocopherols on

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[2] To whom requests for reprints should be addressed. Present address: Training Center for Public Health Research, Box 2067, Hagerstown, MD 21742-2067.

[3] Unless otherwise specified, "serum" will include both plasma and serum.

Table 1 Summary of previous findings regarding within-individual variation of serum concentrations of carotenoids, retinol, α-tocopherol, and ascorbic acid

	Ref. no. of the study										
	2	3	4	5	6	7	8	9	10	3	11
Period of study	Day	Day	2 wk	2 wk	Menstrual cycle	Menstrual cycle	Season 6 mo	Season 5–9 mo	Season 12 mo	1 yr	22 yrs
Index of variation	CV[a]	CV	CCC	CCC	CV	CV	ICC	Difference	CV	CV	PMC
No. of subjects	15	33	23	23	30	12	92	66	18	29	442
α-Carotene		0.09	0.95		0.09	0.01			0.47	0.31	
β-Carotene	0.06	0.08	0.92		0.05	0.04	0.81[b]	50%	0.36	0.20	0.43
Cryptoxanthin		0.08	0.91		0.06				0.37	0.20	
Lutein/zeaxanthin		0.09	0.59		0.10	0.05			0.29	0.18	
Lycopene			0.89		0.06	0.05			0.43	0.18	
Total carotenoids			0.68			0.04					
Retinol	0.04			0.81		0.05	0.70		0.14		
α-Tocopherol	0.05						0.57[c]	No difference	0.11		0.47
Ascorbic acid							0.29				0.22

[a] CV, coefficient of variation; CCC, concordance correlation coefficient; ICC, intraclass correlation coefficient; PMC, product-moment correlation coefficient.
[b] α- and β-carotene.
[c] α- and γ-tocopherol.

serum from 260 individuals selected as normal controls (12).[4] These individuals had donated blood for a serum bank in 1974 and again in 1989. This study compares the assay results for both time periods by rank order correlation and also compares these coefficients with those for blood pressure determinations done at the times of blood donation.

Materials and Methods

From August through November, 1974 and again from May through November, 1989, community-wide programs were conducted in Washington County, Maryland to collect blood for a serum bank and subsequent cancer research. Mobile trailers were moved to many different parts of the county to allow all segments of the adult population an opportunity to participate. Approximately one-third took part in the 1974 program (CLUE I) and a similar proportion participated in the 1989 program (CLUE II).

In 1974, a brief history was first taken. After the several minutes required for this procedure, blood pressure was measured by trained nurses using a mercury sphygmomanometer with the subject seated. Diastolic pressures were defined as the point of disappearance of the Korotkov sounds. The lowest of three determinations was recorded. Finally, blood was drawn into a 15-ml vacutainer (Becton Dickinson, Rutherford, NJ), allowed to clot at room temperature for 30 min, and then refrigerated at 4°C until the serum was separated, usually within 3–4 h. The serum was kept at −70°C until aliquots for assays were prepared by thawing in ice water under dim yellow light. The assay specimens were shipped to the laboratory under dry ice and kept there at −70°C until assayed.

In 1989, the procedures were similar. The only major difference was that blood was collected in 20-ml vacutainers containing heparin (Becton Dickinson) and kept at 4°C until the plasma was separated 2–6 h later. The plasma was kept frozen at −70°C until aliquoted, shipped to the laboratory, and assayed. For the present study, specimens from CLUE I and CLUE II were aliquoted, shipped, and assayed similarly at the same time.

To study repeatability over time, assay results from previous studies were used from controls matched to the breast, lung, or prostate cancer cases who had donated blood in 1974 and again in 1989. Serum and plasma from breast and prostate cancer controls were handled similarly and assayed for carotenoids, retinoids, and tocopherols by high-performance liquid chromatography (13, 14). For lung cancer controls, the high-performance liquid chromatographic procedure differed slightly (15). For the breast and prostate cancer controls, assays were performed by the Department of Biomedical Research, Our Lady of Mercy Medical Center (Bronx, NY) in 1995 and 1998, respectively; assays of specimens from lung cancer controls were performed at the Department of Epidemiology, School of Public Health, University of Minnesota (Minneapolis, MN) in 1995.

It was not appropriate to compare only the absolute concentrations of the various micronutrients in the 1974 and 1989 paired specimens. The 1974 specimens were sera, whereas the 1989 specimens were plasma; storage times were markedly different for serum and plasma; and the subjects were 15 years older in 1989 with concomitant changes in lifestyle. Because assays for the breast, lung, and prostate cancer controls were done at different times and in different laboratories, results for participants in these three studies were analyzed separately by Spearman's rank order correlation coefficients (16). Systolic and diastolic blood pressure values were treated similarly to obtain their rank order correlation coefficients.

Results

Paired results of serum and plasma assays were available for 64 breast cancer controls, 30 lung cancer controls, and 166 prostate cancer controls (Table 2). As expected from the types of cancer to which controls were matched, most subjects for the present study were males. Breast cancer controls were younger than the other two groups, of whom two-thirds were more than 65 years of age in 1989. Most of the controls were still married. Lung cancer controls were somewhat better educated than the breast or prostate cancer controls. Only 13.0% of the total group had changed smoking habits between 1974 and 1989 (they had quit smoking during that period).

The mean concentrations of the various analytes, which are shown in Table 3, differed among the three groups of controls, presumably because of differences in the populations, storage times, and assay locations. There was also considerable variation in the percentage differences in the mean values between the 1974 and 1989 specimens. The mean difference for all analytes was −2.9% (SD, 15.4). Disregarding sign, the mean percentage difference was 11.7% (SD, 10.1).

Mean systolic blood pressures were remarkably similar at the two times. Mean diastolic pressures varied somewhat more, decreasing by 5.8%, 5.9%, and 7.7% among breast, lung, and prostate controls, respectively.

Spearman rank order correlations are shown in Table 4. For the various analytes, the mean value was 0.445 with a SD

[4] K. J. Helzlsouer, personal communication.

Table 2 Percentage distribution of controls for cases of cancer of the breast, lung, and prostate, by status in 1989

Characteristics	Matched controls for cases of cancer of		
	Breast (n = 64)	Lung (n = 30)	Prostate (n = 166)
Sex			
Male	0	46.7	100.0
Female	100.0	53.3	0
Age (yrs)			
<65	53.1	33.3	33.1
65–74	35.9	46.7	54.2
75+	10.9	20.0	12.7
Marital status			
Married	65.6	60.0	89.8
Not married[a]	34.4	40.0	10.2
Years of school completed			
<12	35.9	20.0	39.8
12	50.0	53.3	40.4
13+	14.1	27.7	19.9
Smoking history[b] (1974 and 1989)			
Never (1974 & 1989)	53.1	46.7	27.7
Former (1974 & 1989)	14.1	26.7	51.8
Smoker (1974, former 1989)	12.4	20.0	12.0
Smoker (1974 & 1989)	20.4	6.7	8.4

[a] Single, separated, divorced, widowed.
[b] Smoking includes cigarettes, pipes, and cigars.

of 0.106. Two coefficients were less than 0.30: (*a*) α-carotene among lung cancer controls; and (*b*) retinyl palmitate among prostate cancer controls. Two coefficients were greater than 0.60: (*a*) cryptoxanthin among lung cancer controls; and (*b*) α-tocopherol among prostate cancer controls. For only two coefficients, α-carotene and lycopene among lung cancer controls, did the 95% confidence limits include 0. For blood pressure determinations, the mean rank order correlation coefficient was 0.46. None of the confidence limits included 0.

To see whether seasonal variations affected the rank order correlations, four pairs of blood collection months were selected: (*a*) September 1974 and spring (May and June) 1989; (*b*) October and November 1974 and spring 1989; (*c*) October and November 1974 and summer (July and August) 1989; and (*d*) September through November in both years. Rank order correlation coefficients were calculated for each of the micronutrients and for each of the seasonal period pairs. No clear cut differences in the rank order coefficients by season were observed, although there was a suggestion that they were least for carotenoid concentrations in serum collected in September 1974 and again in spring 1989. Repeatability might also have been affected because marital or smoking status changes between 1974 and 1989 might have been associated with dietary changes. However, such status changes were too few to allow meaningful comparisons to be made.

The failure of mean blood pressure levels to increase with the aging of the study population stimulated a closer look at the data. Of the 260 participants in this study, 67 who were not taking blood pressure medication in 1974 were doing so in 1989. Their systolic and diastolic blood pressures decreased by an average of 9.4 and 9.5 mm Hg, respectively. Regression to the mean also played a part. Among persons not taking blood pressure medication at either time, those with systolic pressures over 150 mm Hg in 1974 showed an average decrease in systolic pressure of 15.4 mm Hg, whereas those with systolic pressures less than 130 mm Hg increased by only 7.2 mm. Diastolic pressures showed a similar phenomenon: readings more than 95 mm decreased by an average of 14.3 mm, whereas 1974 readings of less than 80 mm Hg were 1.6 mm higher in 1989. It is also possible that apprehension about participating in a blood donation program was less on the second experience than it had been initially and that this might have affected blood pressure levels.

Discussion

It should be obvious that the population for this study is a selected one. First, to have donated blood in 1974 and 1989 required not only a survival of 15 years but also the ability to be ambulatory at each time. To have volunteered to give blood on two occasions for scientific study also sets these participants apart from the two-thirds who did not donate blood. Comparisons with the 1975 private census and the 1990 official Census showed that the participants tended to be female, in the 50–70-year age group, better educated, and nonsmokers. To the extent that the participants were more likely to maintain the same lifestyle and dietary habits from 1974 to 1989 than nonparticipants, the results in this study may overemphasize repeatability. We know of no feasible way to check this possibility. It should also be remembered that giving blood was not entirely an act of volunteering in support of a good cause. In 1974, many persons avowedly participated to have their blood pressures checked, and in 1989, an even higher proportion came to have a free cholesterol assay.

For a number of reasons, the micronutrient concentrations in specimens collected in 1974 and 1989 could not be expected to be the same in absolute terms. Serum was collected in 1974, and plasma was collected in 1989. However, many persons with high concentrations at one time were still in the group with high concentrations later on. For this reason, it seems reasonable to assess serum micronutrient concentrations as risk factors for disease in terms of their relative concentrations assessed by rank order correlation coefficients of assay results of specimens from the same individuals done at different times.

Although the rank order correlation coefficients for the micronutrients assayed in specimens taken in 1974 and 1989 are only moderately high (mean, 0.445; SD, 0.106), their magnitude is similar to those for the blood pressure readings at the same times (mean, 0.457; SD, 0.089). Blood pressure determinations from the 1974 project have been shown to be significantly predictive of subsequent coronary disease and increased atherosclerotic changes in the carotid arteries (17). This study involved the 1702 Washington County residents who took part in the 1974 blood collection program and who, as participants in the Atherosclerosis Risk in Communities study, were given a thorough cardiovascular examination in the period 1987 through 1989 (18). That examination included a history of previous cardiovascular disease and an ultrasound examination of both carotid arteries. Persons were classified as hypertensive in 1974 if their systolic blood pressures were 140 mm Hg or higher or if their diastolic blood pressures were 90 mm Hg or higher. The relative risk of having had a heart attack or coronary artery surgery between 1974 and 1989 associated with this classification of hypertension was 2.2 (95% confidence interval, 1.3–3.5). Similarly, among persons with no history of coronary disease or stroke before 1989, the hypertensives in 1974 had a relative risk of 2.0 (95% confidence interval, 1.4–2.8) of having increased subclinical atherosclerosis of the carotid arteries in 1989–1992.

These findings and the similarity of rank order correlation coefficients between micronutrients and blood pressure suggest that measurements of various micronutrients from a single sample can provide useful risk factors for disease processes in which they are suspected of playing a part. To confirm this possibility, future studies should report within-person variability in terms of rank order in addition to absolute values.

Table 3 Mean serum concentrations in 1974 and percentage differences of 1989 values from those of 1974 for specified micronutrients and for blood pressures among matched controls for breast, lung, and prostate cancer cases, Washington County, Maryland

	Breast (n = 64)			Lung (n = 30)			Prostate (n = 166[a])		
	1974	% Difference[b]	P	1974	% Difference	P	1974	% Difference	P
Micronutrients (units)									
Total carotenoids (μg/dl)	105.4	−4.6	0.36				63.3	−2.4	0.47
α-Carotene (μg/dl)	4.5	−21.9	0.26	4.2	7.4	0.54	3.1	18.8	0.01
β-Carotene (μg/dl)	19.8	−25.5	<0.01	22.4	−2.4	0.84	11.4	−5.3	0.51
Cryptoxanthin (μg/dl)	13.2	−1.4	0.89	9.4	−11.1	0.22	7.8	5.1	0.48
Lutein/zeaxanthin (μg/dl)	27.5	−7.0	0.27	22.1	−19.0	<0.01	14.8	−12.2	<0.01
Lycopene (μg/dl)	40.8	−14.8	0.14	41.9	−9.1	0.33	40.1	17.2	<0.01
α-Tocopherol (mg/dl)	1.31	7.3	0.18	0.78	9.0	0.17	1.34	8.3	0.02
γ-Tocopherol (mg/dl)	0.25	−4.3	0.38				0.26	20.6	<0.01
Retinol (μg/dl)	59.0	9.8	<0.01				67.9	4.0	0.01
Retinyl palmitate (μg/dl)	12.8	−49.1	<0.01				11.5	7.2	0.39
Blood pressure									
Systolic (mm Hg)	131.4	−0.8	0.67	139.2	0.1	0.95	140.5	−2.5	0.02
Diastolic (mm Hg)	82.6	−5.8	<0.01	84.9	−5.9	<0.01	88.1	−7.7	<0.01

[a] n for α-carotene is 143, and n for retinyl palmitate is 140.
[b] % Difference of 1989 mean concentration from that of 1974.

Table 4 Spearman rank order correlation coefficients (rho) between 1974 and 1989 values for selected micronutrients and for blood pressures among matched controls for breast, lung, and prostate cancer cases, Washington County, Maryland

	Breast		Lung		Prostate	
	rho	95% CI[a]	rho	95% CI[a]	rho	95% CI[a]
Micronutrients (units)						
Total carotenoids (μg/dl)	0.48	0.25–0.65			0.46	0.32–0.57
α-Carotene (μg/dl)	0.48	0.26–0.65	0.25	−0.13–0.57	0.37	0.22–0.51
β-Carotene (μg/dl)	0.46	0.24–0.64	0.54	0.21–0.76	0.52	0.40–0.63
Cryptoxanthin (μg/dl)	0.51	0.30–0.68	0.66	0.39–0.83	0.46	0.32–0.57
Lutein/zeaxanthin (μg/dl)	0.46	0.24–0.64	0.46	0.10–0.71	0.44	0.30–0.56
Lycopene (μg/dl)	0.32	0.07–0.53	0.35	−0.02–0.64	0.35	0.20–0.48
α-Tocopherol (mg/dl)	0.46	0.23–0.64	0.46	0.11–0.71	0.61	0.50–0.70
γ-Tocopherol (mg/dl)	0.53	0.32–0.69			0.48	0.34–0.59
Retinol (μg/dl)	0.35	0.10–0.55			0.58	0.46–0.67
Retinyl palmitate (μg/dl)	0.32	0.08–0.53			0.21	0.04–0.37
Blood pressure						
Systolic (mm Hg)	0.47	0.22–0.63	0.62	0.32–0.80	0.39	0.24–0.51
Diastolic (mm Hg)	0.38	0.14–0.57	0.47	0.12–0.71	0.41	0.28–0.54

[a] CI, confidence interval.

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REPORTS

Association Between Glutathione *S*-Transferase M1, P1, and T1 Genetic Polymorphisms and Development of Breast Cancer

*Kathy J. Helzlsouer, Ornella Selmin, Han-Yao Huang, Paul T. Strickland, Sandra Hoffman, Anthony J. Alberg, Mary Watson, George W. Comstock, Douglas Bell**

Background: **Glutathione *S*-transferases (GSTs) are encoded by a superfamily of genes and play a role in the detoxification of potential carcinogens. In a nested case–control study, we investigated associations between genetic variability in specific GST genes (GSTM1, GSTT1, and GSTP1) and susceptibility to breast cancer.** *Methods:* **In 1989, a total of 32 898 individuals donated blood samples to a research specimen bank established in Washington County, MD. Genotypes of blood specimen DNA were determined for 110 of 115 women with incident cases of breast cancer diagnosed during the period from 1990 through 1995 and up to 113 of 115 control subjects. Associations between specific genotypes and the development of breast cancer were examined by use of logistic regression to calculate odds ratios (ORs) and 95% confidence intervals (CIs).** *Results:* **The GSTM1 homozygous null genotype was associated with an increased risk of developing breast cancer (OR = 2.10; 95% CI = 1.22–3.64), principally due to an association with postmenopausal breast cancer (OR = 2.50; 95% CI = 1.34–4.65). For GSTP1, the data were suggestive of a trend of increasing risk with higher numbers of codon 105 valine alleles (compared with isoleucine alleles); a 1.97-fold increased risk of breast cancer (95% CI = 0.77–5.02) was associated with valine/valine homozygosity. The risk of breast cancer associated with the GSTT1 homozygous null genotype was 1.50 (95% CI = 0.76–2.95). The risk of breast cancer increased as the number of putative high-risk genotypes increased (*P* for trend <.001) (OR = 3.77; 95% CI = 1.10–12.88 for a combined genotype of GSTM1 null, GSTT1 null, and either GSTP1 valine heterozygosity or GSTP1 valine homozygosity).** *Conclusions:* **Our findings suggest that genetic variability in members of the GST gene family may be associated with an increased susceptibility to breast cancer.** **[J Natl Cancer Inst 1998;90:512–8]**

A large proportion of breast cancer cases cannot be attributed to known risk factors. Further insight into the etiology of breast cancer may be gained by identifying susceptibility factors that predispose individuals to breast cancer if they are exposed to particular environmental agents. An example of such candidate susceptibility factors would include inherited differences in carcinogen metabolism as observed for the *N*-acetyltransferases and glutathione S-transferases (GSTs). A report *(1)* supporting this model suggests that low-activity *N*-acetyltransferase genotypes may predispose women to breast cancer induced by cigarette smoking. The GSTs are a superfamily of genes whose gene products catalyze the conjugation of reactive chemical intermediates to soluble glutathione *(2)*. Of particular interest, GSTM1 and GSTP1 can detoxify carcinogenic polycyclic aromatic hydrocarbons, such as benzo[*a*]pyrene and the mycotoxin aflatoxin, while GSTT1 can detoxify smaller reactive hydrocarbons, such as ethylene oxide and diepoxybutane *(3)*. In addition, glutathione transferases may have a role in the metabolism of lipid and DNA products of oxidative stress *(4,5)* and also in the resistance to cancer chemotherapeutic agents *(6)*.

In humans, hereditary differences in specific GST enzyme activities are due to genetic polymorphisms *(7,8)*. GSTM1 enzyme activity is absent in about 45%–50% of Caucasian populations *(8–13)*. The absence of GSTM1 activity is caused by inheritance of two null alleles (alleles that have a deletion of the GSTM1 gene); similarly, individuals with no GSTT1 activity also have inherited null alleles of the GSTT1 gene. The GSTM1 null genotype has been associated with an increased risk of lung, bladder, and colon cancers *(5,8–11,14)*. The GSTT1 null genotype has been associated with an increased risk of ulcerative colitis *(15)*, colorectal cancer *(16)*, and myelodysplastic syndromes *(17)*. A coding sequence polymorphism, A313G (changing codon 105 from Ile to Val), in the GSTP1 gene was identified several years ago *(18)*, but little information has been published about how it affects GSTP1 phenotype in human tissues or its association with cancer risk. Zimniak et al. *(19)* reported that the GSTP1 105 Val allele had reduced catalytic activity when expressed in *Escherichia coli*, and recent experiments from our laboratory *(20)* suggest that the 105 Val allele was associated with lower GSTP1 enzyme activity in lung tissue samples. The GSTP1 enzyme can mediate the detoxification of numerous chemicals including chemotherapy agents (e.g., alkylating agents). Increased expression of GSTP1 in tumors has been hypothesized to play a role in the drug resistance seen

**Affiliations of authors:* K. J. Helzlsouer, H.-Y. Huang, S. Hoffman, A. J. Alberg, G. W. Comstock (Department of Epidemiology), P. T. Strickland (Department of Environmental Health Sciences), The Johns Hopkins School of Hygiene and Public Health, Baltimore, MD; O. Selmin, M. Watson, D. Bell, Genetic Risk Group, Laboratory of Computational Biology and Risk Assessment, National Institute of Environmental Health Sciences, Research Triangle Park, NC.

Correspondence to: Kathy J. Helzlsouer, M.D., M.H.S., Department of Epidemiology, The Johns Hopkins University School of Hygiene and Public Health, 615 N. Wolfe St., Baltimore, MD 21205.

See "Notes" following "References."

in many cancers, and this phenomenon has been observed in cancers of the breast, head and neck, and skin and in acute leukemia *(21–23)*. Because of GSTP1's potential role in detoxifying carcinogenic compounds, it is plausible that individuals with GSTP1 105 Val alleles may be at increased risk of cancer from exposure to chemicals detoxified by the GSTP1 enzyme. Harries et al. *(24)* found an association between GSTP1 105 Val homozygosity and a risk of bladder and testicular cancers, but they observed no statistically significant association with breast cancer risk. The study by Harries et al. compared a cancer case patient series with an infirmary-based control group and provided no information on basic characteristics of the control groups such as age and sex. In a study of men with lung cancer, hydrophobic DNA adduct levels were higher among the patients with the GSTM1 genotype and heterozygous or homozygous for the 105 Val GSTP1 allele *(25)*. Future investigations would benefit from stronger study designs.

To investigate the association between genetic variability in GSTM1, GSTT1, and GSTP1 and risk of breast cancer, a nested case–control-subject study was conducted by use of a specimen bank established in Washington County, MD, in 1989.

Subjects and Methods

Study Population

In 1989, a research specimen bank was established with 32 898 individuals donating a blood sample after they signed an informed consent. Of the participants, 25 081 (14 625 women) were residents of Washington County and formed the study cohort. Compared with the Washington County population, the cohort participation rates were higher among women and older individuals. Incident breast cancer cases (n = 115) occurring during the period from 1990 through 1995 were identified by linkage of the study cohort to the Washington County Cancer Registry. Eleven of the 115 case patients had ductal carcinoma *in situ*. The registry identifies case patients from discharge records of the Washington County Hospital, the only hospital in the county, and from death certificates. To estimate completeness of ascertainment of the Washington County Cancer Registry, we compared the number of cancer cases obtained through the registry with the number reported to the Maryland Cancer Registry for Washington County for a 1-year period. The Maryland Cancer Registry has mandatory reporting of incident breast cancer cases from hospitals, pathology laboratories, and physicians throughout Maryland *(26)*. The first complete report of the state registry was for 1993. In that year, 89 cases of breast cancer were recorded in the Washington County Cancer Registry, and 81 incident cases were reported to the Maryland Cancer Registry.

Each case patient was matched to one control subject on age (within 1 year), race (all were white), menopausal status, time from last menstrual period, and date of blood donation within 2 weeks.

At the time of blood donation, participants completed a brief questionnaire that ascertained smoking status, height, weight, and medication taken in the previous 48 hours. As part of a larger study on environmental risk factors and breast cancer, case patients and control subjects were sent a self-administered questionnaire in 1995 to obtain more detailed information about risk factors for breast cancer. Of the 115 case patients, 104 (90.4%) returned the completed questionnaire; of the 115 control subjects, 103 (89.6%) returned the completed questionnaire. Exposure to such factors as hormone replacement therapy among case patients and control subjects was truncated at the date of diagnosis of the case patient.

Laboratory Assays

Blood was collected in 20-mL heparinized tubes and centrifuged at 1500*g* for 30 minutes at room temperature within 6 hours of collection. Plasma, buffy coat, and red blood cells were separated and stored at −70 °C within 24 hours of collection. The buffy coat was kept frozen until it was thawed for DNA extraction for this study.

DNA was extracted from the thawed white blood cell fraction from each study subject by high-salt fractionation *(27)* followed by chloroform–isoamyl alcohol extraction *(28)*. The concentration of DNA was adjusted to 100 μg/mL, and the DNA was stored at −70 °C until genotype analysis. DNA of sufficient quality for all three genotype analyses was successfully extracted from 110 of 115 case patients and up to 113 of 115 control subjects. GSTM1 and GSTT1 genotypes were determined by use of the multiplex polymerase chain reaction (PCR) method of Chen et al. *(17)*. This technique does not distinguish between heterozygote and homozygote GSTM1- or GSTT1-positive genotypes, but it conclusively identifies null genotypes. The GSTP1 (Ile105Val) genotype was determined by use of the PCR–restriction fragment length polymorphism method of Watson et al. *(20)*. Briefly, genomic DNA (50 ng) was added to a PCR mix of GSTP1 primers 2306F (5′-GTA GTT TGC CCA AGG TCA AG) and 2721R (5′-AGC CAC CTG AGG GGT AAG) (15 pmol each), deoxynucleoside triphosphates (200 μmol each), 0.5 U *Taq* polymerase (Amplitaq; The Perkin-Elmer Corp., Norwalk, CT) in buffer composed of 16.6 m*M* $(NH_4)_2SO_4$, 50 m*M* β-mercaptoethanol, 6.8 μ*M* ethylene diaminetetraacetic acid (EDTA), 67 μ*M* Tris, 80 μg/mL bovine serum albumin, and 2.0 m*M* $MgCl_2$. The mixture was assembled at 85 °C and placed in a Perkin-Elmer 9600 thermocycler at 94 °C. After an initial denaturation step at 94 °C for 3 minutes, five cycles of PCR were carried out (cycle 1: 94 °C for 15 seconds, 64 °C for 30 seconds, and 72 °C for 60 seconds) during which the annealing temperature decreased by 1 °C for each cycle *(20)*. This step was followed by 25 cycles of amplification at 94 °C for 15 seconds, 59 °C for 30 seconds, and 72 °C for 60 seconds and a final extension at 72 °C for 5 minutes. The high initial annealing temperature (64 °C) followed by decreasing temperatures in the PCR program was found to reduce the appearance of nonspecific amplification products *(20)*. PCR products were digested for 4–16 hours with the restriction enzyme *Alw*26I, which distinguishes between the restriction sites on the Ile allele (ACA TCT) and the Val allele (ACG TCT.)

Products of PCR amplification or restriction digests were analyzed by gel electrophoresis, and genotypes were determined by characteristic band patterns on the gel. The GSTP1-genotyping method has primers that flank an *Alw*26I restriction site that is present in all samples, thus providing a control cut for the restriction digest portion of the assay *(20)*.

To ensure laboratory quality control, two independent readers interpreted the gel photographs. Any sample with ambiguous results (generally due to low PCR yield) was retested, and a random selection of 10% of all samples was repeated. No discrepancies were discovered upon replicate testing.

Statistical Analysis

The association between the GSTM1, GSTT1, and GSTP1 genotypes and the development of breast cancer was examined by use of conditional and unconditional logistic regression analyses to calculate the odds ratios (ORs) and 95% confidence intervals (CIs). The GST assays place individuals into distinct categories: those with present or null genotypes for GSTM1 and GSTT1 and those with homozygous Ile/Ile or with heterozygous or homozygous 105 Val allele for GSTP1. ORs and 95% CIs were estimated for each of these categories with the present genotype (GSTM1 and GSTT1) or homozygous Ile/Ile (GSTP1) designated as the referent category.

Of interest was the relationship between the GST genotypes and the development of breast cancer within categories of risk factors to assess for the presence of confounding and interaction. The specific variables used in these stratified analyses were menopausal status of the case patient at the time of diagnosis, smoking history, history of alcohol consumption, body mass index (BMI), ever use of hormone replacement therapy, and family history of breast cancer in mother, sister, or grandmother. For most of these variables, stratified analyses required breaking the match to preserve adequate numbers in the strata. Consequently, ORs presented were estimated by use of unconditional logistic regression, adjusting for the matching factors of age and menopausal status at the time of blood donation. For the analyses in which both could be estimated, unmatched analyses produced results similar to those produced by the matched analyses. Logistic regression analysis adjusting for known or suspected risk factors for breast cancer (age at menarche and first birth, family history, BMI, alcohol consumption, exogenous hormone use, and smoking history) did not alter the point estimates of the ORs; therefore, only unadjusted analyses or analyses adjusting only for matching factors are presented.

Women with breast cancer were classified as postmenopausal at diagnosis on the basis of their response to questionnaire items concerning the date of their last menstrual period as well as history of hysterectomy and oophorectomy. Women with information missing in regard to their last menstrual period or women with a history of hysterectomy

without oophorectomy were considered to be postmenopausal if they were diagnosed after the age of 51 years (the median age of menopause).

Tests for interaction were based on the *P* value (two-sided) of the likelihood ratio test for adding the interaction term (genotype by stratified variable) to the model that already included the main effect variable (genotype), stratified variable, age, and menopausal status at baseline.

Results

The association between presumed risk factors and the development of breast cancer is shown in Table 1. Case patients and control subjects were closely matched on age and menopausal status at the time of blood donation and had similar ages at menarche and at first birth. A family history of breast cancer in a grandmother, mother, or sister was associated with a 2.25-fold increase in breast cancer risk. Ever use of oral contraceptives was similar for case patients and control subjects. Fewer case patients than control subjects reported ever using hormone replacement therapy, but the difference was not statistically significant. Neither cigarette smoking nor alcohol intake was associated with the development of breast cancer. Twenty-eight case patients were premenopausal at the time of diagnosis.

The risk of breast cancer associated with GST genotypes, stratified by menopausal status at the time of diagnosis of the case patient, is shown in Table 2. The null GSTM1 genotype was associated with a 2.1-fold increase in the risk of developing breast cancer (OR = 2.10; 95% CI = 1.22–3.64). After stratification by menopausal status at the time of diagnosis, the association was apparent only among women with postmenopausal breast cancer (OR = 2.50; 95% CI = 1.34–4.65). The GSTT1 null genotype was associated with a 1.5-fold increased risk of developing breast cancer, but this association was not statistically significant. For GSTP1, a trend of increasing risk for Ile/Val heterozygotes and Val/Val homozygotes was suggested but was not statistically significant (*P* for trend = .10). Risks were similar among premeno-

Table 1. Selected characteristics for breast cancer case patients (n = 115) and matched control subjects (n = 115), Washington County, MD, 1989

Characteristic	Case patients*	Control subjects*	Matched odds ratio (95% confidence interval)	*P*
Age, y, in 1989, mean ± standard deviation†	60.4 ± 11.7	60.2 ± 11.5	—	.91
Age, y, at menarche, mean ± standard deviation†,‡	12.6 ± 1.5	12.8 ± 1.5	—	.21
Age, y, at first birth, mean ± standard deviation†,§	21.7 ± 3.9	22.5 ± 4.3	—	.23
Menopausal status at blood donation†				
Premenopausal	28 (24.3)	26 (22.6)	—	.76
Postmenopausal	87 (75.7)	89 (77.4)	—	
Family history of breast cancer in mother, sister, or grandmother				
No	84 (73.0)	99 (86.1)	1.0 (referent)	
Yes	31 (27.0)	16 (13.9)	2.25 (1.14–4.44)	
Body mass index, kg/m^2				
≤24.47	47 (40.9)	58 (50.4)	1.0 (referent)	
>24.47	68 (59.1)	57 (49.6)	1.61 (0.89–2.90)	
History of cigarette smoking				
Never	75 (65.2)	72 (62.6)	1.0 (referent)	
Former	27 (23.5)	24 (20.9)	1.07 (0.56–2.06)	
Current	13 (11.3)	19 (16.5)	0.66 (0.30–1.44)	
No. of cigarettes smoked per day‖				
1–10	17 (14.8)	13 (11.3)	1.02 (0.44–2.37)	
11–20	10 (8.7)	10 (8.7)	1.03 (0.36–2.97)	
≥20	4 (3.5)	2 (1.7)	1.51 (0.25–9.27)	
Missing data	9 (7.8)	18 (15.7)		
History of alcohol drinking				
Never	53 (46.1)	60 (52.2)	1.0 (referent)	
Ever	51 (44.3)	41 (35.7)	1.51 (0.81–2.80)	
Missing data	11 (9.6)	14 (12.2)		
No. of alcoholic drinks consumed per week				
<1	20 (17.4)	13 (11.3)¶	1.13 (0.42–3.08)	
1–3	16 (13.9)	16 (13.9)	1.18 (0.51–2.73)	
≥4	15 (13.0)	9 (7.8)	1.72 (0.68–4.47)	
Use of hormone replacement therapy				
Never	72 (62.6)	63 (54.8)	1.0 (referent)	
Ever	24 (20.9)	27 (23.5)	0.79 (0.41–1.53)	
Missing data	19 (16.5)	25 (21.7)		
Use of oral contraceptives				
Never	75 (65.2)	74 (64.3)	1.0 (referent)	
Ever	28 (24.3)	26 (22.6)	1.1 (0.5–2.2)	
Missing data	12 (10.4)	15 (13.0)		

*Unless otherwise specified, values in columns = number of case patients or control subjects (%).

†Matching factors.

‡Data available for 100 case patients and 98 control subjects.

§Data available for 84 case patients and 86 control subjects.

‖Among former and current smokers.

¶Numbers do not sum to 41 because of missing data on number of drinks consumed per week.

Table 2. Association between GSTM1, GSTT1, and GSTP1 genotypes and development of breast cancer according to menopausal status at time of diagnosis of the case patient*

Genotype	Total			Premenopausal at diagnosis			Postmenopausal at diagnosis		
	Case patients	Control subjects	OR† (95% CI)	Case patients	Control subjects	OR† (95% CI)	Case patients	Control subjects	OR† (95% CI)
GSTM1									
Present	39	60	1.0 (referent)	9	9	1.0 (referent)	30	51	1.0 (referent)
Null	71	52	2.10 (1.22–3.64)	15	16	1.0 (0.29–3.45)	56	36	2.50 (1.34–4.65)
Missing data	5	3		1	0		4	3	
GSTT1									
Present	80	88	1.0 (referent)	17	20	1.0 (referent)	63	68	1.0 (referent)
Null	30	24	1.50 (0.76–2.95)	7	5	1.50 (0.42–5.32)	23	19	1.50 (0.67–3.34)
Missing data	5	3		1	0		4	3	
GSTP1									
Ile/Ile	41	56	1.0 (referent)	6	9	1.0 (referent)	35	47	1.0 (referent)
Ile/Val	54	48	1.48 (0.81–2.73)	17	13	1.98 (0.44–8.81)	37	35	1.33 (0.68–2.61)
Val/Val	15	9	1.97 (0.77–5.02)	1	3	0.54 (0.04–6.67)	14	6	2.71 (0.91–8.03)
Missing data	5	2		1	0		4	2	
			P for trend = .10			*P* for trend = 1.0			*P* for trend = .08

*GST = glutathione *S*-transferase; OR = odds ratio; CI = confidence interval; null = alleles that have a deletion of the GSTM1 or GSTT1 genes.
†Matched ORs from conditional logistic regression.

pausal and postmenopausal breast cancer case patients for the Ile/Val genotypes, but the trend of increasing risk from Ile/Val heterozygotes to Val/Val homozygotes was observed only among women with postmenopausal breast cancer (*P* for trend = .08).

To explore possible gene–environment interactions, we examined the association between genotype and breast cancer risk stratified by selected characteristics (Table 3). Because the risk associated with the null GSTM1 genotype was observed only among postmenopausal breast cancer case patients, in Table 3 the results for GSTM1 are shown stratified by menopausal status at the time of diagnosis of the case patient. While statistical power to detect gene–environment interaction was limited in this study, significant interactions were observed between GSTM1 and BMI and between GSTT1 and a history of alcohol consumption (Table 3). Postmenopausal women null for GSTM1 and having a BMI above the median (>24.47 kg/m^2) had a sevenfold increase in breast cancer risk, whereas

Table 3. Association between GSTM1, GSTT1, and GSTP1 and development of breast cancer, stratified by selected characteristics*

	Premenopausal at diagnosis, GSTM1		Postmenopausal at diagnosis, GSTM1		Premenopausal and postmenopausal combined			
					GSTT1		GSTP1	
	Present, OR†	Null, OR (95% CI)	Present, OR†	Null, OR (95% CI)	Present, OR†	Null, OR (95% CI)	Ile/Ile, OR†	Ile/Val or Val/Val, OR (95% CI)
Family history of breast cancer in mother, sister, or grandmother								
No	1.0	0.92 (0.26–3.29)	1.0	2.62 (1.30–5.27)	1.0	1.32 (0.64–2.72)	1.0	1.71 (0.92–3.16)
Yes	1.0	—‡	1.0	2.99 (0.68–13.19)	1.0	2.10 (0.45–9.75)	1.0	2.00 (0.55–7.26)
Use of hormone replacement therapy								
Never	1.0	1.88 (0.39–9.65)	1.0	2.22 (0.99–4.97)	1.0	1.67 (0.73–3.78)	1.0	2.06 (1.02–4.15)
Ever	1.0	0.30 (0.01–8.34)	1.0	3.37 (0.91–12.44)	1.0	0.58 (0.15–2.20)	1.0	1.21 (0.37–3.91)
History of cigarette smoking								
Never	1.0	1.15 (0.27–4.84)	1.0	2.41 (1.11–5.23)	1.0	1.24 (0.58–2.67)	1.0	1.51 (0.75–3.04)
Ever	1.0	0.56 (0.06–4.82)	1.0	2.71 (0.93–7.86)	1.0	1.49 (0.51–4.31)	1.0	2.23 (0.90–5.53)
History of alcohol drinking								
Never	1.0	0.82 (0.12–5.34)	1.0	2.58 (1.08–6.14)	1.0	0.94 (0.42–2.11)§	1.0	1.42 (0.65–3.08)
Ever	1.0	0.72 (0.10–5.20)	1.0	2.88 (1.06–7.84)	1.0	5.35 (1.11–25.9)	1.0	2.09 (0.88–5.00)
Body mass index, kg/m^2								
≤24.47	1.0	2.27 (0.35–14.59)	1.0	0.91 (0.37–2.26)	1.0	1.01 (0.39–2.62)	1.0	2.36 (1.04–5.35)
>24.47	1.0	0.60 (0.10–3.51)	1.0	7.02 (2.79–17.66)‖	1.0	1.56 (0.67–3.63)	1.0	1.32 (0.63–2.77)

*GST = glutathione *S*-transferase; OR = odds ratio; CI = confidence interval.
†Referent category.
‡OR could not be estimated because of insufficient numbers in the cell.
§*P* for interaction = .03.
‖*P* for interaction = .002.

women null for GSTM1 and having a BMI less than the median were not at increased risk of breast cancer. Among women with the null GSTT1 genotype, a significant increase in risk was observed only for women who ever reported alcohol intake.

GSTM1, GSTT1, and GSTP1 are involved in detoxification of a variety of compounds, some that overlap between enzymes and some that are highly specific *(2)*. To investigate whether profiles of GST genotypes may be associated with the risk of breast cancer, we examined the risk of breast cancer associated with combinations of genotypes. The reference group consisted of individuals with all three putative low-risk genotypes, i.e., the presence of GSTM1 *and* GSTT1 genotypes and the homozygous Ile/Ile genotype for GSTP1. Individuals heterozygous and homozygous for the Ile105Val allele were combined for this analysis. Table 4 displays the risk of breast cancer associated with each combination of genotypes as well as the trend in risk associated with one, two, or three putative high-risk genotypes. The presence of at least one putative high-risk genotype was associated with an increased risk of breast cancer. The risk of breast cancer increased as the number of putative high-risk genotypes increased (P for trend <.001).

Discussion

The glutathione transferases are involved in the metabolism of a wide variety of potential carcinogenic compounds, and the isozymes have distinct but overlapping substrate specificity. These substrates include polycylic aromatic hydrocarbon diol-epoxides, organic epoxides, peroxides, *N*-acetyl benzoquinoneimine, hydroxyalkyl-arenes, and steroids. Several classes of compounds have been identified as mammary carcinogens in animals [e.g., aromatic amino/nitro compounds and epoxide-forming chemicals *(29)*], and aromatic DNA adducts have been found in higher levels in breast tissue from cancer patients than in breast tissue from control subjects without cancer *(30,31)*. Thus, it is biologically plausible that low-activity-level genotypes would be associated with an enhanced susceptibility to cancer. In this community-based, prospective study, we observed an increased risk for breast cancer associated with the GSTM1 null genotype, GSTP1 105 Val allele genotypes, and the combined GST at-risk genotypes. For GSTP1, the results were suggestive of a linear increase in risk for the heterozygous and homozygous 105 Val genotypes that reflect intermediate and low levels of enzyme activity. These associations did not vary significantly by age of onset of breast cancer, smoking history, alcohol intake, or history of exogenous hormone use. Women who developed breast cancer were about 1.5 times more likely than control subject women to have the GSTT1 null genotype, but this association was not statistically significant.

The significant association that we observed between the null GSTM1 genotype and breast cancer risk is not consistent among three other published reports *(12,13,32)*. Zhong et al. *(32)* reported results of a multi-cancer (breast, bladder, and colorectal cancers), hospital-based study with case patient samples collected from multiple hospitals in the U.K. and control samples collected from other locations in Scotland. No information was provided on the age, sex, menopausal status, clinical status, or any other possible risk factors of either case patients or control subjects. Zhong et al. found a GSTM1 null genotype frequency of 41.8% among control subjects and of 47.7% among breast cancer case patients; however, the difference in proportions was not statistically significant. Ambrosone et al. *(12)* carried out a population-based, case–control study of postmenopausal breast cancer and reported no increased risk associated with the GSTM1 null genotype among the total study group of 216 case patients and 282 community-based control subjects (OR = 1.10; 95% CI = 0.73–1.64). An increased risk associated with the GSTM1 null genotype was observed only among postmenopausal women under the age of 58 years at diagnosis, but this increase was not statistically significant (OR = 2.44; 95% CI = 0.89–6.64). A study of prevalent versus incident cases of breast cancer *(13)* observed a risk of 1.3 (95% CI = 0.91–1.86) associated with the null genotype among prevalent cases and a risk of 1.08 (95% CI = 0.74–1.57) among incident cases. Among prevalent cases, the propor-

Table 4. Association between GST genotype profile and the development of breast cancer*

GSTM1	GSTT1	GSTP1	Case patients (n = 110)	Control subjects (n = 112)	Unmatched OR (95% CI)
		All putative low-risk genotypes			
1. Present	Present	Ile/Ile genotype	12	27	1.0 (referent)
		One putative high-risk genotype			
		Total	36	44	1.90 (0.84–4.29)
2. Null	Present	Ile/Ile	16	20	1.86 (0.72–4.80)
3. Present	Null	Ile/Ile	6	4	3.41 (0.81–14.37)
4. Present	Present	Ile/Val or Val/Val genotype	14	20	1.63 (0.62–4.31)
		Two putative high-risk genotypes			
		Total	52	35	3.53 (1.56–7.97)
5. Null	Null	Ile/Ile	7	5	3.25 (0.85–12.39)
6. Null	Present	Ile/Val or Val/Val	38	21	4.30 (1.79–10.34)
7. Present	Null	Ile/Val or Val/Val	7	9	1.86 (0.55–6.23)
		All three putative high-risk genotypes			
8. Null	Null	Ile/Val or Val/Val	10	6	3.77 (1.10–12.88)†

*GST = glutathione *S*-transferase; OR = odds ratio; CI = confidence interval.

†P for trend for none, one, two, and all three putative high-risk genotypes <.001.

tion of women with breast cancer who had the null genotype increased with increasing duration of survival *(13)*. The primary difference in our study population compared with other study populations was the percentage of case patients with the GSTM1 null genotype. One could speculate that the different results among the studies could be due to differences in exposures relevant to breast cancer.

The GSTP1 family of glutathione transferases is involved in the metabolism of alkylating agents used in chemotherapy, and prior studies have focused on measuring enzyme activity levels in tumor tissues as a potential factor in drug resistance. Harries et al. *(24)* recently analyzed GSTP1 Ile105Val alleles in a control series and in a series of patients who had a variety of cancers. No information was provided on the age, sex, menopausal status, or clinical status of the control group. Harries et al. found the likelihood of GSTP1 Ile105Val heterozygosity to be 1.5 times higher among breast cancer patients than among control subjects (95% CI = 0.79–2.98) and homozygosity for Val/Val to be 1.6 times higher among breast cancer patients than among control subjects (95% CI = 0.42–5.70). A statistically significant increased risk was observed among patients with testicular and bladder cancers. The study by Harries et al., like the previously mentioned study by Zhong et al. *(32)*, suffers from design weaknesses, such as lack of characterization of cancer case patients and control subjects and exploration of potential confounding. The present study has the strength of a prospective study design with information about other risk factors for breast cancer.

Our findings suggest that genetic differences in some members of the glutathione transferase gene family, which code for phase II detoxification enzymes, may be associated with an increased susceptibility to breast cancer. A statistically significant increase in the risk of breast cancer was observed with increasing burden of putative high-risk genotypes. Significant elevations in the risk of cancer are likely to be observed only among those individuals with the susceptibility genotype as well as with a history of the relevant environmental exposure. The differences in results among the reported studies may be due to variations in the study populations and their exposures. As stated previously, the glutathione transferases are involved in the detoxification of many electrophilic substances, only some of which may be relevant to breast carcinogenesis. Thus, the search must be continued to identify the relevant environmental or endogenous exposures involved in the potential gene–environment interactions. A possible relevant endogenous exposure is estrogen and its catechol metabolites of estrogen *(33)*. In support of this hypothesis are two findings. First, we observed statistically significant interaction between the BMI and the GSTM1 genotype and the risk of breast cancer. Obesity is a known risk factor for breast cancer, thought to be due to peripheral conversion of androgens to estrogen *(34)*. Second, catechol-*O*-methyltransferase is involved in the inactivation of catechol estrogen metabolites. When we examined the risk of postmenopausal breast cancer according to catechol-*O*-methyltransferase polymorphism for low activity, we found an increased risk among individuals with either the GSTM1 null genotype or the GSTP1 105-Val homozygous or heterozygous genotype *(35)*. A possible explanation for observing this interaction is the role of glutathione transferases in the metabolisms of the reactive catechol metabolites. Because the suggested high-risk genotypes occur quite commonly, the calculated population attributable risk may be high, even with relatively moderate magnitudes of associated risk. For example, assuming a 44% frequency of the null GSTM1 genotype in the population and an associated twofold increased risk of breast cancer, the percentage of breast cancer cases attributed to the null GSTM1 genotype would be estimated to be 30.5%. If the relevant exogenous exposure that increases risk could be determined and avoided among individuals with these susceptible genotypes, then a substantial proportion of breast cancer cases potentially can be prevented. Such calculations are instructive because they highlight the potential importance of this line of inquiry, but they should be considered premature. These findings need to be replicated in other populations before we can be certain that GST polymorphisms are truly involved in the etiology of breast cancer.

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Notes

Present address: O. Selmin, University of Arizona, Tucson.

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Serum Concentrations of Organochlorine Compounds and the Subsequent Development of Breast Cancer[1]

Kathy J. Helzlsouer,[2] Anthony J. Alberg, Han-Yao Huang, Sandra C. Hoffman, Paul T. Strickland, John W. Brock, Virlyn W. Burse, Larry L. Needham, Douglas A. Bell, Jackie A. Lavigne, James D. Yager, and George W. Comstock

Departments of Epidemiology [K. J. H., A. J. A., H-Y. H., S. C. H., G. W. C.] and Environmental Health Sciences [P. T. S., J. A. L., J. D. Y.], School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland 21205; Division of Environmental Health Laboratory Sciences, Centers for Disease Control, Atlanta, Georgia 30341 [J. W. B., V. W. B., L. L. N.]; and National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709 [D. A. B.]

Abstract

A nested case-control study was conducted to examine the association between serum concentrations of 1,1-dichloro-2,2-*bis*(*p*-chlorophenyl)ethylene (DDE), the primary metabolite of 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT), and polychlorinated biphenyls (PCBs) and the development of breast cancer up to 20 years later. Cases (n = 346) and controls (n = 346) were selected from cohorts of women who donated blood in 1974, 1989, or both, and were matched on age, race, menopausal status, and month and year of blood donation. Analyses were stratified by cohort participation because median DDE and PCB concentrations among the controls were 59 and 147% higher in 1974 than 1989, respectively. Median concentrations of DDE were lower among cases than controls in both time periods [11.7% lower in 1974 (P = 0.06) and 8.6% lower in 1989 (P = 0.41)]. Median concentrations of PCBs were similar among cases and controls [P = 0.21 for 1974 and P = 0.37 for 1989 (Wilcoxon signed rank test)]. The risk of developing breast cancer among women with the highest concentrations of DDE was roughly half that among women with the lowest concentrations, whether based on concentrations in 1974 [odds ratio (OR), 0.50; 95% confidence interval (CI), 0.27–0.89; P_{trend} = 0.02] or in 1989 (OR, 0.53; 95% CI, 0.24–1.17; P_{trend} = 0.08). The associations between circulating concentrations of PCBs and breast cancer were less pronounced but still in the same direction (1974: OR, 0.68; 95% CI, 0.36–12.9; P_{trend} = 0.2; and 1989: OR, 0.73; 95% CI, 0.37–1.46; P_{trend} = 0.6). Adjustment for family history of breast cancer, body mass index, age at menarche or first birth, and months of lactation did not materially alter these associations. These associations remained consistent regardless of lactation history and length of the follow-up interval, with the strongest inverse association observed among women diagnosed 16–20 years after blood drawing. Results from this prospective, community-based nested case-control study are reassuring. Even after 20 years of follow-up, exposure to relatively high concentrations of DDE or PCBs showed no evidence of contributing to an increased risk of breast cancer.

Introduction

The organochlorine compounds DDE[3] (the primary metabolite of DDT) and PCBs are suspected of having a role in breast cancer etiology because: (*a*) they are stored in adipose tissue and found in breast milk; (*b*) some have estrogen-like activity; and (*c*) some are metabolized to highly reactive compounds (1–5). Although banned in the United States in the early 1970s, DDT and PCBs are long-lived compounds that persist in the environment, with diet being the most common route of continued exposure (6–9).

Results of studies that examined the association between organochlorine compounds and breast cancer are summarized in Tables 1A and 1B (10–23). Six of the seven case-control studies based on measures of adipose tissue concentrations found similar or lower concentrations of DDE among cases than controls (10–16); four of five (11–14) observed higher adipose tissue concentrations of PCBs among cases than controls. Four case-control studies used serum organochlorine concentrations as the marker of exposure with blood drawn after the diagnosis of breast cancer (17–20). Two of these studies measured DDE and PCB concentrations (17, 20), and the other two measured only DDE concentrations (18, 19). Wolff *et al.* (17) found elevated levels of serum DDE and PCBs in newly diagnosed breast cancer cases compared with controls participating in the same screening study. DDE exhibited a dose-response relationship with the risk of breast cancer. The relationship between PCBs and breast cancer suggested a possible threshold effect. All of the serum samples of cases were obtained within 6 months of diagnosis of breast cancer. The subsequent studies have not found evidence of significantly higher levels of DDE (18–20) or PCBs (20) among women

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[2] To whom requests for reprints should be addressed, at Department of Epidemiology, The Johns Hopkins University School of Hygiene and Public Health, Room E-6132, 615 North Wolfe Street, Baltimore, MD 21205. Phone: (410) 955-9727; Fax: (410) 614-2632.

[3] The abbreviations used are: DDE, 1,1-dichloro-2,2-*bis*(*p*-chlorophenyl)ethylene; DDT, 1,1,1-trichloro-2,2-*bis*(*p*-chlorophenyl)ethane; PCB, polychlorinated biphenyl; COMT, catechol-*O*-methyltransferase; CLUE I, Campaign against Cancer and Stroke; CLUE II, Campaign against Cancer and Heart Disease; OR, odds ratio; CI, confidence interval; GT, glutathione.

Table 1 Published studies of the association between organochlorine compounds and breast cancer

Study[a]	Year (place)	No. of cases/ controls	Mean concentrations (SD)		OR by categories					P_{trend}
			Cases	Controls	1 (lowest)	2	3	4	5 (highest)	
A. Published studies of the association between DDT compounds and breast cancer										
Case-Control										
Adipose Tissue										
Davies (10)	1975	29/29	5.4 ppm	7.1 ppm						
Wasserman (11)	1976 (Brazil)	9/5	2.7 ppm	6.7 ppm						
Unger (12)	1982 (Denmark)	14/21	1.2 (0.63) ppm	1.2 (0.8) ppm						
Mussalo-Rauhamaa (13)	1985–86 (Finland)	41/33	0.96 (0.6) mg/kg	0.98 (0.9) mg/kg						
Falck (14)	1987 (United States)	20/20	1877 (1283) ng/g[c]	1174 (630) ng/g[c]						
van't Veer (15)	1991–92 (Europe)	265/341	1.35 μg/g	1.51 μg/g	1.0	1.14	0.71	0.48		0.02
Dewailly (16)	1991–92 (Canada)	20/17	ER−[b] 0.6 (0.3) μg/g ER+ 2.1 (2.0) μg/g	0.7 (0.5) μg/g						
Serum										
Wolff (17)	1985–91 (United States)	58/171	11.0 (9.1) ppb	7.7 (6.8) ppb	1.0	1.67	4.37	2.31	3.68	0.04
Schechter (18)	1994 (Vietnam)	21/21	12.2 (2.4) ng/ml	16.8 (4.1) ng/ml	1.0	0.45	1.14			
Lopez-Carillo (19)	1994–96	141/141	562.5 (676.2) ng/g[c]	505.5 (567.2) ng/g[c]	1.0	0.60	0.76			
Moysich (20)	1986–91	154/192	11.5 (10.5) ng/g[c]	10.8 (10.6) ng/g	1.0	1.01	1.34			0.25
Prospective studies, nested case-control										
Serum										
Krieger (21)	1964–71 (United States)	150/150	43.3 (25.9) ppb	43.1 (23.7) ppb	1.0	1.31	1.26			0.43
Hunter (22)	1989–90 (United States)	240/240	6.01 (4.56) ppb[c]	6.97 (5.99) ppb[c]	1.0	0.88	0.56	0.73	0.66	0.22
Hoyer (23)	1976 (Denmark)	240/477	NA		1.0	0.79	0.92	0.84		0.65
B. Published studies of the association between PCBs and breast cancer										
Case-Control										
Adipose Tissue										
Wasserman (11)	1976 (Brazil)	9/5	9.1 ppm	3.0 ppm						
Unger (12)	1982 (Denmark)	14/21	3.9 (1.0) ppm	3.9 (1.3) ppm						
		18/35	6.47 ± 2.35	5.1 (2.4) ppm						
Mussalo-Rauhamaa (13)	1985–86 (Finland)	20/20	1.1 (0.6) mg/kg	1.3 (0.8) mg/kg						
Falck (14)	1987 (United States)	41/33	1669 (894) ng/g	1105 (424) ng/g						
Dewailly (16)	1991–92 (Canada)	20/17	ER− 0.3 (0.7) μg/g ER+ 0.4 (0.1) μg/g	04 (0.2) μg/g						
Serum										
Wolff (17)	1985–91 (United States)	58/171	8.0 ± 4.1 ppb	6.7 ± 2.9 ppb	1.0	5.18	7.02	4.10	4.35	0.16
Moysich (20)	1986–91	154/192	4.3 (2.4) mg/g[c]	4.1 (2.2) mg/g	1.0	0.70	1.14			0.51
Prospective studies, nested case-control										
Serum										
Krieger (21)	1964–71 (United States)	150/150	4.4 (1.8) ppb	4.8 (2.5) ppb	1.0	1.17	0.94	0.55	0.59	0.9
Hunter (22)	1989–90 (United States)	240/240	5.1 (2.5) ppb[c]	5.2 (2.3) ppb	1.0	0.57	0.54			0.26
Hoyer (23)	1976 (Denmark)	240/477	NA		1.0	0.92	0.78	1.11		0.77

[a] Number in parentheses cites reference.
[b] ER−, estrogen receptor negative; ER+, estrogen receptor positive; NA, not available.
[c] Lipid-adjusted.

with breast cancer compared with control women, with the exception of one study that found an association among parous women who never breast-fed (20). Two of these studies were conducted in Mexico and Vietnam where DDT is actively used (18, 19).

Three prospective studies using nested case-control study designs have examined the association between DDE and PCBs and subsequent development of breast cancer (21–23). The study by Krieger *et al.* (21) in California examined concentrations in blood samples drawn between 1964 and 1971 from 150 women who went on to develop breast cancer and 150 matched control women. The development of breast cancer was not associated with serum concentrations of DDE or PCBs regardless of race, year of diagnosis, length of follow-up, or estrogen receptor status (21). Hunter *et al.* (22) examined concentrations of organochlorine compounds in blood samples drawn in 1989 or 1990 among 240 women who developed breast cancer by 1992 and matched control women. The risk of breast cancer tended to be lower among women with higher serum concentrations of DDE and PCBs but the trends were not statistically significant. The exposure assessment in this study preceded the breast cancer but only by a maximum period of 3 years. Høyer *et al.* measured concentrations of organochlorine compounds in blood samples obtained in 1976 from 240 women who developed breast cancer by 1995 and 477 matched control women (23). The development of cancer was not associated with DDE, total DDT, or total PCB concentrations.

We used the resources of two specimen banks established in Washington County, Maryland in 1974 and 1989 to prospectively examine the association between exposure to DDE and PCBs and the development of breast cancer through 1994. The 1974 cohort provides a long follow-up period, and the serum

concentrations were likely to be maximal in 1974, close to the time organochlorine compounds were banned in the United States. The 1989 cohort was used to examine the association between more current organochlorine concentrations and breast cancer.

The susceptibility to exogenous exposures such as organochlorine compounds may vary by levels of detoxification enzymes such as glutathione transferases. Glutathione-μ, encoded by *GSTM1* genotype, is known to catalyze the conjugation of glutathione with oxidative intermediates of a variety of carcinogenic compounds. Studies in rodents indicate that oxidative intermediates of organochlorines are excreted in urine and feces as conjugates (24–26), and other organochlorine compounds—3,4-dichloro-4-nitrobenzene and 1-chloro-2,4-dinitrobenzene—are known to be a substrate for GT-μ and other GT family enzymes (27). We recently reported an association between putative high-risk genotypes for *GSTM1*, *GSTP1*, and *GSTT1* and the development of breast cancer (28). There is also a suggestion of an interaction between *COMT* activity and *GSTM1* null genotype and the risk of breast cancer (29). The present study examines the association between organochlorine compounds and the risk of breast cancer, stratifying by genotype among women who participated in the 1989 cohort.

Materials and Methods

From August through November 1974, the Campaign against Cancer and Stroke was conducted in Washington County, MD. Referred to as CLUE I (from the slogan, "Give us a Clue to Cancer and Stroke"), it was designed to collect blood samples from as many adults as possible to provide specimens for a serum bank. A total of 25,802 persons donated blood, of whom 20,305 were county residents. Linkage of the records from this program to those of a private census in the summer of 1975 indicated that almost one-third of the adult population of the county had participated. Participation was best in the age group 35–65 years and was slightly better among females, the better-educated, and nonsmokers. A brief history form was completed at the time of blood collection. Blood was drawn into 15-ml Vacutainers (Becton Dickinson), and serum aliquots were stored at −70°C.

The Campaign against Cancer and Heart Disease (CLUE II) was similar to CLUE I and was conducted from May through October 1989. Brief histories and blood pressures were taken, and 20 ml of blood were drawn into heparinized Vacutainers (Becton Dickinson). Plasma, buffy coat, and 1.8 ml of packed red cells were stored at −70°C. A total of 32,892 persons participated, of whom 25,080 were Washington County residents. A total of 8395 residents participated in both CLUE I and CLUE II campaigns. Comparisons with published figures from the 1990 Census indicated that again approximately 30% of adult residents had participated. As before, women and the better educated had higher than average participation rates, as did the age group 45–70 years.

Cases were drawn from participants who were residents of Washington County, Maryland who donated blood for the serum bank in 1974 only (CLUE I), in 1989 only (CLUE II), or in both programs (CLUE I and II). The cases were women who were first diagnosed as having breast cancer (ICD9, 174) after having donated blood for one of the two CLUE programs. Cases were identified by linkage of the cohort participants to the Washington County Cancer Registry. Registry cases were identified from discharge records of the Washington County Hospital, the only hospital in the county, and from death certificates. Through June 1994, 346 women were identified who had developed breast cancer after donating blood, had no other invasive cancers, and had serum available. One hundred ninety-nine women had local disease; of these, 23 were *in situ* ductal carcinoma, 109 had regional disease, and 26 were of unknown stage. The majority of women (295) had participated in CLUE I; 115 women had participated in CLUE II, and 64 of these had also donated blood to CLUE I. Participants who had donated to both CLUE programs developed their breast cancer after the CLUE II program (1989) and were included in each program-specific analysis. Completeness of ascertainment was estimated by comparing the number of breast cancer cases reported to the Maryland Cancer Registry (in operation since 1992) to the number of cases obtained through the Washington County Registry for 1993 (30). In that year, 90 cases of breast cancer were recorded in the Washington County Cancer Registry and 81 of these were reported to the Maryland Cancer Registry.

Controls were selected from participants who were residents of Washington County when they donated blood and were not diagnosed with an invasive cancer (with the possible exception of basal or squamous cell cancer of the skin) at the time when cases were diagnosed. Each case was matched to one control by sex (all were women), race (all were Caucasian), age (within one year), menopausal status, date of blood donation, and the CLUE programs in which they participated. Among premenopausal women, cases and controls were also matched by the day of the menstrual cycle at the time of blood donation.

One case-control set from CLUE II was eliminated because the chosen control had been diagnosed with breast cancer; 26 sets were excluded from CLUE I because of inadequate quantity of sera for assays; and 34 sets from CLUE I and 10 sets from CLUE II were eliminated because either the case or the control sera failed to meet quality control standards for added marker compounds during the assay. Thus, data on organochlorine compounds were available for 235 matched sets from CLUE I and 105 matched sets from CLUE II.

Questionnaire Data

At the time of blood donation, participants completed a brief questionnaire that ascertained smoking status, height, weight, and medication use in the previous 48 h. Women with breast cancer and matched controls (or their next of kin, if deceased) were sent a self-administered questionnaire in 1995 to obtain more detailed information about breast cancer risk factors. Eighty-nine percent of cases and 76% of control women returned the questionnaires. Of the returned questionnaires, 31 (10.6%) were answered by surrogates of cases and 14 (5.3%) were answered by surrogates of controls. Exposure to factors such as the use of hormone replacement therapy and alcohol intake was truncated at the date of diagnosis of the case for both cases and controls.

Laboratory Assays

DDE and PCBs. Blood was collected in red-topped Vacutainer tubes in 1974 and in heparinized green-topped Vacutainer tubes in 1989. Serum or plasma was prepared from blood samples within 24 h of collection and usually within 6 h (note the term serum will be used generally to denote serum from CLUE I and plasma from CLUE II specimens.) In 1989, plasma, buffy coat, and RBCs were separated and stored at −70°C within 24 h of collection. In that year, 90 cases of breast cancer were recorded in the Washington County Cancer Registry, and 81 of these were reported to the Maryland Cancer Registry.

DDE and PCBs in sera were assayed using solid-phase extraction followed by gas chromatography with electron capture detection as described previously by Brock *et al.* (31).

Plasma samples were assayed using liquid/liquid extraction and adsorption chromatography. The method was similar to that described by Burse *et al.* (32) with the following exceptions: (*a*) only one elution fraction was collected from Florisil [6% ethyl ether/petroleum ether that contained pesticides (except Dieldrin and Endrin) and PCBs]; (*b*) the acid wash of the eluted fraction was omitted; and (*c*) the eluted fraction was not further eluted through silica gel. The eluted fraction was assayed by gas chromatography with electron capture detection per Brock *et al.* (31). PCBs congeners were referred to by a standard numbering scheme as described by Ballschmitter and Zell (33). Samples were simultaneously assayed for total cholesterol and triglyceride levels, and lipid-adjusted values were calculated using the formula described by Philips *et al.* (34).

Thirty-one quality-control sample sets consisting of pooled sera were included, with one quality-control set approximately every 10th case-control set. One laboratory quality-control sample consisting of spiked bovine serum and one reagent blank were also assayed with every batch of ten samples. If either of these samples were of the normal controls limits, the samples were reassayed. Twenty-three of the CLUE I serum samples were reextracted for repeat assays. In these instances, the matched sample in the set was not reassayed at the same time. All of the sets were included in the final analysis because separate analyses with these case-control sets excluded were similar to the overall findings. Quality-control samples of pooled sera were used to calculate coefficients of variation. Intra-assay coefficients of variations for 1974 serum samples were 9.4% for total PCBs and 14.7% for DDE; for 1989, the coefficients were 17.4% for total PCBs and 8.4% for DDE. Interset coefficients of variations for 1974 serum samples were 19.7% for total PCBs and 12.8% for DDE; for 1989, the coefficients were 20.0% for PCBs and 6.7% for DDE.

Genotyping. The buffy coat was kept frozen until thawed for DNA extraction for this study. DNA was extracted from the thawed WBC fraction from each study subject by high-salt fractionation (35) followed by chloroform/isoamyl alcohol extraction (36). Concentration of DNA was adjusted to 100 μg/ml and stored at −70°C until genotype analysis. *GSTM1*, *GSTT1*, and *GSTP1* genotype could be determined for 110 cases and 113 controls. *GSTM1* and *GSTT1* genotypes were determined using the multiplex PCR method of Chen *et al.* (37), which does not distinguish between heterozygote and homozygote *GSTM1* or *GSTT1* positive genotypes but conclusively identifies null genotypes. *GSTP1* (Ile105Val) genotype was determined using the PCR-RFLP method of Watson *et al.* (38). *CYP17* genotype could be determined on 109 cases and 113 controls. The *CYP17* genotype was determined using the PCR-RFLP method of Carey *et al.* (39), in which restriction digestion by *Msp*AI identifies the presence of the *A2* allele. *COMT* genotype was determined by a PCR-based RFLP assay (29) for 112 matched case-control pairs.

Statistical Analyses

Statistical anlayses were performed separately for CLUE I and CLUE II cohort participants and for lipid-adjusted and -unadjusted DDE and PCB data. For CLUE I, total DDE was defined as the sum of *o,p'*DDT + *p,p'*DDT + *o,p'*DDE + and *p,p'*DDE; total PCB was defined as the sum of the following measured PCB congeners: 28, 52, 56, 74, 101, 105, 110, 118, 138, 146, 153, 156, 170, 172, 177, 178, 180, 183, 187, 189, 193, 194, 195, 201, 203, and 206. For CLUE II, total DDE was defined as the sum of *o,p'*DDE, and *p,p'*DDE; total PCB was defined as the sum of the following measured PCB congeners: 52, 66, 101, 105, 110, 118, 138, 146, 153, 156, 170, 172, 177, 178, 180, 183, 187, 189, 193, 195, 201, and 203. Differences in definition of total DDE and PCBs between the two cohorts reflect the inability to detect certain congeners and metabolites in the 1989 samples, in which overall concentrations were lower compared with 1974.

Concentrations of DDE and total PCBs between cases and controls were compared using the Wilcoxon signed-rank test. Conditional logistic regression models were used to assess the association between concentrations of DDE and PCBs and the risk of developing breast cancer. Concentrations of DDE and PCBs were categorized into fifths (CLUE I) or thirds (CLUE II) based on the distributions in the control groups. Thirds were used for CLUE II because of the number of observations for that program. ORs and the corresponding 95% CIs for each upper fifth (or third) compared with the lowest fifth (or third) were calculated. Adjustment for known or suspected risk factors for breast cancer other than the matching variables, such as family history of breast cancer, body mass index at age 20 or current, age at menarche, age at first birth, and duration of lactation, did not alter the point estimates of ORs. Therefore, only unadjusted analyses are presented. Trend tests were performed by conditional logistic regression analyses using median values of each fifth or third category among the controls as the independent variable.

Additional analyses included examining the association by individual PCB congener, the PCB congeners that contributed to most of the total, by structure-activity groups as proposed by Wolff *et al.* (40), and by strata of menopausal status at diagnosis, years since diagnosis, history of lactation (among parous women), estrogen receptor status, and genotype of *GSTM1*, *GSTT1*, *GSTP1*, *CYP17*, and *COMT*. Conditional logistic regression analyses were used in these analyses except for the stratification analyses of history and estrogen receptor status, in which unconditional logistic regression adjustment for matching factors (age and menopausal status at baseline) were used to preserve adequate numbers in the strata. Women with breast cancer were classified as postmenopausal at diagnosis based on the response to the questionnaire regarding the date of last menstrual period as well as the history of hysterectomy and oophorectomy. Women with missing information regarding the last menstrual period or women with a history of hysterectomy without oophorectomy were considered to be postmenopausal if diagnosed at age 51 or older. A two-sided P less than 0.05 was considered statistically significant.

Results

Selected characteristics of cases and controls are presented in Table 2. Cases and controls were closely matched on age. The distribution of risk factors were in the direction expected based on previous research with cases more likely than controls to have a family history of breast cancer, later age at first birth or nulliparity, earlier age at menarche (CLUE II only), higher education, higher weekly consumption of alcoholic drinks, and fewer months of breast feeding. Only the associations with family history of breast cancer ($P = 0.01$) and >12 months of lactation ($P = 0.01$) were statistically significant. Mean (SD) for cholesterol concentrations were: 204.5 (48.6) for cases and 217.5 (54.7) for controls in the CLUE I study and 180.3 (38.4) for cases and 189.6 (39.0) for controls in the CLUE II study.

Table 2 Characteristics of cases and controls by cohort participation

	1974		1989	
	% cases (*N* = 235)	% controls (*N* = 235)	% cases (*N* = 105)	% controls (*N* = 105)
Age at donation				
≤40	20.9	20.4	3.8	2.9
41–50	23.8	26.0	21.0	22.9
51–60	33.6	31.9	18.1	15.2
≥61	21.7	21.7	57.1	59.1
Education				
<12 years	38.3	43.0	24.8	31.4
≥12 years	61.7	57.0	75.2	68.6
Family history of breast cancer (define: mother, sister, grandmother)				
No	79.2	91.1	73.3	87.6
Yes	20.9	8.9	26.7	12.4
Age at first birth				
<20	18.7	19.2	22.9	22.9
20–29	40.4	35.7	51.4	49.5
≥30	6.8	4.3	1.0	3.8
Nulliparous	11.9	10.2	13.3	9.5
Missing	22.1	30.6	11.4	14.3
Age at menarche				
<12	13.2	14.5	19.1	14.3
12–13	40.4	31.9	54.3	49.5
≥14	17.5	17.9	16.2	22.9
Missing	28.9	35.7	10.5	13.3
Lactation (months)				
None	51.9	40.0	61.9	47.6
1–12	16.6	14.9	19.1	20.0
>12	6.0	9.8	5.7	17.1
Missing	25.5	35.3	13.3	15.2
Hormone replacement therapy				
Never	58.3	48.5	65.7	54.3
Ever	16.6	14.9	20.0	24.8
Missing	25.1	36.6	14.3	21.0
Alcohol consumption (no. of drinks/week)				
Never	48.1	46.8	47.6	50.5
<1	12.8	8.1	17.1	12.4
1–3	7.7	8.1	14.3	15.2
≥4	13.2	7.2	14.3	7.6
Missing	18.3	29.8	6.7	14.3
Cigarette smoking (at donation)				
Never	60.0	57.5	64.8	63.8
Former	16.6	15.7	23.8	21.0
Current	23.4	26.8	11.4	15.2
Time to diagnosis				
<2			48.6	
3–5 years	12.3	NA[a]	51.4	NA
6–10	17.9			
11–15	28.5			
≥16	41.3			
Estrogen receptor status				
ER-negative	11.5	NA	19.0	NA
ER-positive	33.2		66.7	
Unknown	55.3		14.3	

[a] NA, not applicable; ER, estrogen receptor.

Mean (SD) for triglyceride concentrations were: 152.3 (64.0) for cases and 153.6 (69.3) for controls in the CLUE I study and 149.6 (60.1) for cases and 148.2 (66.7) for controls in the CLUE II study.

Mean and median concentrations of DDE and total PCBs, unadjusted and adjusted for serum lipid concentrations, are shown in Table 3. In both CLUE I and CLUE II, prediagnostic mean and median concentrations of DDE were lower among cases than controls. PCB levels were similar for cases and controls. The concentrations of both organochlorine compounds were higher in 1974 than in 1989. For example, when considering the concentrations without lipid adjustment, the median values of the control groups in 1974 were 59% higher for DDE and 147% higher for PCBs.

The risk of breast cancer did not increase with increasing serum concentrations of DDE or PCBs (Table 4). In fact, the risk of breast cancer tended to be lowest among women with the highest concentrations of DDE or PCB. The associations were closer to the null when concentrations were adjusted for lipid levels but provided no evidence of increased risk.

The association between PCBs and breast cancer was further investigated by performing congener-specific analyses, combining the main PCBs (for 1974, PCBs 28, 74, 118, 138, 153, 156, 170, and 180; for 1989, PCBs 118, 138, 153, 170, 180, 189, 195, 201, and 203) and according to structure-activity groups proposed by Wolff *et al.* (40). None of the individually measured congeners were associated with a statistically significant increased risk of breast cancer, and, in general, the risk of breast cancer tended to be lower among women with higher concentrations of the congeners (data not shown). Results of the analysis limited to the main PCBs were similar to the results with total PCBs. Three structure-activity groupings of PCB congeners have been proposed (40). Group 1 congeners are potentially estrogenic (Group 1A: weak phenobarbital inducers, estrogenic, not persistent; Group 1B: weak phenobarbital inducers, persistent); Group 2 congeners are potentially antiestrogenic and immunotoxic, dioxin-like (Group 2A: non-*ortho* and mono-*ortho* substituted; Group 2B: di-*ortho* substituted); and Groups 3 are neither estrogenic nor antiestrogenic but induce enzymes (CYP1A, CYP2B, and phenobarbital). The risk of breast cancer tended to decrease with increasing concentrations of Group 3 congeners. None of the structure-activity groupings were associated with an increased risk of breast cancer (data not shown).

Analysis of the association by time from blood sampling to the time of diagnosis was conducted to assure that the results are not influenced by the presence of occult disease at the time of blood drawing. The results for the different follow-up intervals were consistent with the overall findings. Even for cases diagnosed 16–20 years after blood sampling, the risk of breast cancer among the highest third of the distribution of DDE compared with the lowest third was 0.37 (95% CI, 0.18–0.78); for PCBs, it was 0.51 (95% CI, 0.25–1.05).

Because of the estrogen-like activity of some organochlorine compounds, analyses were performed considering both menopausal status at the time of diagnosis and tumor estrogen receptor status. Tumor estrogen receptor status was available for 86% of cases from the 1989 cohort but for only 45% of the cases from the 1974 cohort because routine tumor estrogen receptor assays were not performed until after 1980. The risk of breast cancer in association with concentrations of DDE and PCBs varied slightly by menopausal status at diagnosis and tumor estrogen receptor status (Table 5). Whether the breast cancer cases were diagnosed when pre- or postmenopausal or with an estrogen receptor-negative or -positive tumor, there was no evidence of a consistent increase in risk with higher levels of organochlorine components. The only significant trend in risk was in the inverse direction among women from the 1974 cohort with postmenopausal onset of breast cancer.

We examined the association with DDE and PCBs stratifying by lactation history among parous women because of previously reported associations between PCBs and DDE and

Table 3 Mean and median DDE and PCB concentrations according to cohort participation

	1974					1989				
	Mean (SD)		Median		P*	Mean (SD)		Median		P*
	Cases	Controls	Cases	Controls		Cases	Controls	Cases	Controls	
Total DDE										
Unadjusted (ng/ml)	11.5 (7.1)	13.6 (10.6)	9.8	11.1	0.06	7.9 (6.4)	9.7 (3.6)	6.4	7.0	0.41
Lipid-adjusted ng/gm	1698.9 (929.3)	1920.3 (1409.0)	1463.9	1668.4	0.20	1311.9 (1036.5)	1586.3 (1557.4)	1119.2	1181.7	0.56
Total PCBs										
Unadjusted (ng/ml)	4.9 (3.8)	4.7 (2.3)	3.9	4.2	0.21	2.1 (2.0)	2.2 (1.9)	1.6	1.7	0.37
Lipid-adjusted (ng/gm)	735.3 (644.8)	663.6 (322.5)	595.4	607.3	0.48	327.7 (306.3)	332.9 (279.6)	252.0	270.3	0.58

* Wilcoxon signed rank test.

Table 4 Association between DDE and PCBs and breast cancer risk

	1974					1989				
	Fifths	Cases	Controls	OR	(95% CI)	Thirds	Cases	Controls	OR	(95% CI)
Total DDE										
Unadjusted ng/ml	1. <6.93	62	47	1.0		1. <4.34	29	35	1.0	
	2. 6.94–9.60	52	47	0.88	(0.52–1.50)	2. 4.35–10.53	59	34	1.80	(0.97–3.33)
	3. 9.61–12.86	48	46	0.83	(0.49–1.43)	3. 10.54–57.16	17	36	0.53	(0.24–1.17)
	4. 12.87–17.74	43	48	0.70	(0.40–1.22)					
	5. 17.75–80.33	30	47	0.50	(0.27–0.89)					
				(P_{trend} = 0.02)					(P_{trend} = 0.08)	
Lipid-adjusted ng/g	1. <1017.19	49	47	1.0		1. <816.3	38	35	1.0	
	2. 1,017.20–1,425.39	61	47	1.24	(0.72–2.13)	2. 816.4–1,595.1	44	35	1.18	(0.65–2.13)
	3. 1,425.40–1,864.57	47	47	0.96	(0.55–1.67)	3. 1,595.2–10,065.6	23	35	0.58	(0.29–1.17)
	4. 1,864.58–2,446.69	42	47	0.86	(0.49–1.51)					
	5. 2,446.70–10,795.91	36	47	0.73	(0.40–1.32)					
				(P_{trend} = 0.13)					(P_{trend} = 0.15)	
Total PCBs										
Unadjusted ng/ml	1. <2.83	53	47	1.0						
	2. 2.84–3.71	56	47	1.07	(0.61–1.89)	1. <1.30	42	34	1.0	
	3. 3.72–4.77	44	47	0.80	(0.44–1.47)	2. 1.31–2.17	29	35	0.65	(0.33–1.30)
	4. 4.78–6.28	42	47	0.75	(0.42–1.34)	3. 2.18–14.63	34	36	0.73	(0.37–1.46)
	5. 6.29–32.60	40	47	0.68	(0.36–1.29)					
				(P_{trend} = 0.16)					(P_{trend} = 0.56)	
Lipid-adjusted ng/g	1. <394.47	42	47	1.0						
	2. 394.48–558.72	59	47	1.41	(0.79–2.50)	1. 13.6–191.8	40	34	1.0	
	3. 558.73–669.46	41	47	0.94	(0.49–1.77)	2. 191.9–333.5	32	35	0.78	(0.41–1.47)
	4. 669.47–852.22	45	47	1.08	(0.59–2.01)	3. 333.6–2,007.9	33	36	0.76	(0.38–1.51)
	5. 852.23–6,460.04	48	47	1.12	(0.59–2.15)					
				(P_{trend} = 0.44)					(P_{trend} = 0.60)	

breast cancer risk among parous women who never lactated (20). The risk of breast cancer was not increased among parous women; in fact, ORs were in the inverse direction with increasing concentrations of DDE and PCBs (data not shown).

The association between concentrations of DDE and total PCBs and the development of breast cancer among CLUE II participants was examined stratifying by polymorphisms of *GSTM1*, *GSTT1*, *GSTP1*, *COMT*, and *CYP17* (Table 6). Associations did not vary significantly by genetic polymorphisms.

Discussion

The results of this prospective study provide evidence against the hypothesis that organochlorine compounds are associated with the risk of developing breast cancer. Women with the highest serum concentrations of DDE had the lowest risk of developing breast cancer. This association held for both unadjusted and lipid-adjusted concentrations and was strongest among women with the longest follow-up interval (16–20 years). The risk of developing breast cancer also tended to decrease with increasing levels of PCBs. Our study has the advantage of being community-based, assessing exposure to DDE and PCBs years before the diagnosis of breast cancer, and examining exposure from two time periods.

We used cohorts from two time periods (1974 and 1989) and, thus, were able to examine the long-term effect of exposure to relatively high levels of the organochlorine compounds present in the population near the time that the compounds were banned as well as more recent, lower levels of exposure. The concentrations of DDE in 1974 were more than twice that observed in Mexico (19), where DDT is still used. Even among women exposed to high levels, we did not observe an increased risk of breast cancer from DDE or PCBs after a follow-up of up to 20 years.

Our study is the largest published to date, and the results are consistent with three previously published nested case-control studies (21–23). Krieger *et al.* examined serum specimens obtained before the banning of the DDE and PCBs. Mean

Table 5 Association between organochlorine components and the risk of breast cancer by menopausal status at diagnosis and estrogen receptor status

	1974				1989			
	Lower third	Middle third	Upper third	P_{trend}	Lower third	Middle third	Upper third	P_{trend}
DDE								
Premenopausal at diagnosis	1.0	0.42	0.86	(0.68)	1.0	4.28	1.42	(0.8)
Postmenopausal at diagnosis	1.0	0.75	0.52	(0.003)	1.0	1.57	0.50	(0.15)
Estrogen receptor-negative	1.0	1.10	1.67	(0.41)	1.0	0.74	0.17	(0.13)
Estrogen receptor-positive	1.0	0.86	0.80	(0.61)	1.0	2.28	0.59	(0.19)
PCB								
Premenopausal at diagnosis	1.0	0.65	2.21	(0.12)	1.0	1.45	2.12	(0.40)
Postmenopausal at diagnosis	1.0	0.67	0.62	(0.10)	1.0	0.83	0.74	(0.44)
Estrogen receptor-negative	1.0	1.13	1.34	(0.71)	1.0	0.19	0.07	(0.06)
Estrogen receptor-positive	1.0	0.98	0.83	(0.69)	1.0	0.91	1.19	(0.55)

Table 6 Association between organochlorine compounds and breast cancer according to genotype, CLUE II, 1989[a]

Genotype	DDE				PCBs			
	Low	Middle	High	P_{trend}	Low	Middle	High	P_{trend}
GSTM1								
Present	1.0	3.23	1.27	0.83	1.0	0.87	1.32	0.53
Null	1.0	1.49	0.27	0.01	1.0	0.59	0.50	0.16
TI								
Present	1.0	2.32	0.52	0.14	1.0	0.79	0.62	0.28
Null	1.0	1.20	0.51	0.36	1.0	0.19	0.93	0.78
PI								
Ile/Ile	1.0	4.20	0.52	0.30	1.0	0.64	0.58	0.34
Ile/Val or Val/Val	1.0	0.94	0.40	0.08	1.0	0.49	0.75	0.74
COMT								
HH	1.0	0.76	0.35	0.22	1.0	0.58	0.92	0.90
HL	1.0	1.76	0.63	0.44	1.0	1.07	0.48	0.17
LL	1.0	3.93	0.59	0.35	1.0	0.19	0.54	0.80
CYP17								
A1/A1	1.0	2.64	0.47	0.17	1.0	0.54	0.35	0.11
A1/A2	1.0	2.30	1.08	0.96	1.0	0.38	1.21	0.49
A2/A2	1.0	0.86	0.05	0.04	1.0	0.84	0.49	0.36

[a] Unconditional logistic regression adjusted for age and menopausal status at baseline.

levels were similar among cases and controls, and there was no evidence of a trend in risk with increasing serum concentrations. Hunter *et al.* compared DDE and PCB concentrations in breast cancer cases diagnosed within 3 years after blood collection in 1989 and 1990 to matched controls. Similar to our results, the risk was lowest among women in the highest category of DDE and PCB concentrations. A nested case-control study from Denmark found no association with concentrations of DDE or PCBs measured from blood samples collected in 1976 (23).

Our findings are also consistent with three of the four studies that have examined the association between serum concentrations of DDE and breast cancer measured after the diagnosis of breast cancer (18–20). Only the study by Wolff *et al.* (17) observed an association between the presence of breast cancer and higher DDE concentrations. Two of these studies also examined the association between concentration of PCBs and breast cancer. Results of the study by Wolff *et al.* (17) were suggestive of a threshold effect of PCBs. Moysich *et al.* (20) observed an increased risk of postmenopausal-onset breast cancer associated with total PCB concentrations and moderately chlorinated PCB congeners only among parous women who never lactated. We were not able to reproduce this observation in our study.

A potential concern in interpreting studies based on serum rather than adipose tissue measurement of exposure to DDE and PCBs is that serum measurements may not adequately reflect adipose tissue levels. Nevertheless, studies have shown good correlation between serum levels and adipose tissue levels (1, 41), and lipid-adjustment of serum is a good estimation of adipose tissue levels (34). In addition, the results of recent serum-based studies are consistent with the majority of published case-control studies involving DDE or PCB exposure based on adipose tissue measurements (Table 1; Refs. 10–16). The largest study published to date of adipose levels of DDE and breast cancer (265 cases, 341 controls) also found a lower risk of breast cancer for women in the highest *versus* the lowest fourth of the DDE distribution (OR, 0.73; 95% CI, 0.44–1.21); PCBs were not assayed (15).

Although we previously reported an association between breast cancer and the putative high-risk polymorphisms of *GSTM1*, *GSTT1*, *GSTP1*, and *COMT*, we found no evidence that these genetic factors influenced susceptibility to organochlorine compound effects. Thus, the search to identify the relevant environmental or endogenous exposures involved in the potential gene-environment interactions related to breast carcinogenesis must continue.

Of the three prospective studies of the association between exposure to DDE and PCBs, two studies examined levels before, or at the time of, banning of compounds and had a long

length of follow-up to the time of diagnosis. None of the three observed an excess risk of breast cancer among women with higher concentrations of DDE or PCBs. The balance of data from both case-control and prospective studies are reassuring that exposure to DDE and PCBs do not confer an increased risk of breast cancer. It now seems likely that whatever environmental exposures contribute to the risk of breast cancer, exposure to DDT and PCBs can be ruled out.

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Household exposure to passive cigarette smoking and serum micronutrient concentrations

Anthony J Alberg, Joyce C Chen, Hongxin Zhao, Sandra C Hoffman, George W Comstock, and Kathy J Helzlsouer

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Anthony J Alberg, Joyce C Chen, Hongxin Zhao, Sandra C Hoffman, George W Comstock, and Kathy J Helzlsouer

ABSTRACT

Background: The associations observed between passive smoking and adverse health outcomes have generated controversy. In part, this could be because the diets of passive smokers, like those of active smokers, differ from those of persons who are not exposed to cigarette smoke, especially with regard to antioxidants.

Objective: Our objective was to assess the relation between household exposure to passive smoking and serum concentrations of retinol, tocopherols, and carotenoids.

Design: A cross-sectional study was conducted in Washington County, MD, to compare exposure to passive smoking at home, recorded in a private census of county residents in 1975, with micronutrient concentrations assayed in serum collected in 1974. This comparison was possible for 1590 control subjects in nested case-control studies conducted between 1986 and 1998.

Results: Among persons who were not current smokers, those who lived with smokers tended to have lower serum total carotenoid, α-carotene, β-carotene, and cryptoxanthin concentrations than did those who lived in households with no smokers. There was little evidence that exposure to passive smoking was associated with reduced serum concentrations of lutein and zeaxanthin, lycopene, retinol, α-tocopherol, or γ-tocopherol.

Conclusions: Among nonsmokers, exposure to passive smoking tended to be associated with lower serum concentrations of the carotenoids most strongly associated with active smoking (total carotenoids, α-carotene, β-carotene, and cryptoxanthin). The associations were weaker for passive smoking than for active smoking. The consistency of the associations observed for active and passive smoking indicates that exposure to passive smoking may result in decreased circulating concentrations of selected micronutrients. *Am J Clin Nutr* 2000;72:1576–82.

KEY WORDS Passive smoking, environmental tobacco smoke, cigarette smoking, α-carotene, β-carotene, cryptoxanthin, lycopene, lutein, zeaxanthin, retinol, α-tocopherol, γ-tocopherol

INTRODUCTION

Persons who smoke cigarettes are known to differ from persons who never smoked with respect to several lifestyle behaviors, including eating less healthful diets and drinking more alcohol (1–5). The same may hold true, to a lesser degree, for comparisons of nonsmokers exposed to passive smoking with nonsmokers who are not exposed to passive smoking. A specific reason for obtaining more information on this topic is to assist in the interpretation of studies of the health effects of passive smoking. Since the initial observation of an association between passive smoking and lung cancer in 1981 (6, 7), passive smoking has been studied widely to assess its potential harmful health effects. Passive smoking has been judged to be causally associated with lung cancer (8) and may be linked with ischemic heart disease (9). Studies of the health effects of passive smoking often compared disease risk among nonsmokers who lived with a smoker (usually their spouse) with the risk among nonsmokers who did not live with any smokers. This design has been criticized on the grounds that households in which smoking takes place may differ from households with no smokers with respect to factors that are associated with disease risk other than passive smoking (10–12).

See corresponding editorial on page 1421.

In the most extreme case, this set of circumstances could theoretically lead to the appearance of passive smoking being associated with adverse health outcomes when, in fact, the association was due to dietary differences rather than to passive smoking. A small proportion of the studies conducted to assess the health effects of passive smoking accounted for diet; for example, 8 of 39 studies reviewed by Hackshaw et al (13) for lung cancer risk controlled for diet.

For these reasons, the relation between passive smoking and dietary factors is worth clarifying. Several studies, summarized in **Table 1**, addressed this question. A common finding was lower fruit, vegetable, and micronutrient intakes (15, 16, 18, 23) by persons exposed to passive smoking relative to those not exposed to passive smoking. The specific micronutrients associated with lower intake have included β-carotene or carotene (14, 15, 19, 23), retinol (15, 16), vitamin C (15–17, 22), and α-tocopherol (16). Only 2 studies in Table 1 looked at serum concentrations of micronutrients, a better indicator of actual cell exposures to nutrients than dietary intake histories. Tribble et al (22) found

[1] From the Department of Epidemiology, The Johns Hopkins School of Hygiene and Public Health, Baltimore.

[2] AJA is a recipient of a Preventive Oncology Academic Award from the National Cancer Institute (CA73790). GWC is a recipient of a Research Career Award from the National Heart, Lung, and Blood Institute (HL21670).

[3] Address reprint requests to AJ Alberg, Department of Epidemiology, Room E6132B, The Johns Hopkins School of Hygiene and Public Health, 615 N Wolfe Street, Baltimore, MD 21205. E-mail: aalberg@jhsph.edu.

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TABLE 1
Summary of studies of the association between passive smoking and dietary factors[1]

Reference and year	Location	Number exposed: not exposed	Exposure assessment criteria	Dietary assessment method	Main findings for persons exposed compared with those not exposed
Sidney et al (14), 1989	California	356:1786	h/wk	Questionnaire	β-Carotene intake lower
LeMarchand et al (15), 1991	Hawaii	40:42	Cotinine/creatinine	Interview	Intake of β-carotene, vitamin A, vitamin C, various vegetables and fruit, cholesterol, and fat lower Intake of tomatoes and oranges higher
Thornton et al (12), 1994	England	933:2387	Any household smoking	Interview	Intake of fruit, salads, and cereal lower Intake of alcohol, fried foods, and bread higher
Emmons et al (16), 1995	United States	2147:1473	Lived with smoker	Questionnaire	Intake of vitamin A, vitamin C, α-tocopherol, retinol, fruit and vegetables, fiber, and iron lower Intake of fat higher
Matanoski et al (17), 1995	United States	2423:1473	Ever married to smoker	Interview or questionnaire	Intake of vitamin C and carbohydrates lower Intake of alcohol and beef higher
Koo et al (18), 1997	Hong Kong Sweden Japan United States	111:419 18:69 4901:8146 84:60	Spouse currently smoking	Interview or questionnaire	Intake of milk (HK, S) and vegetables (S) lower Intake of meat (HK, S), alcohol (HK), fried or grilled food (S), and fat and cholesterol (US) higher
Steenland et al (19), 1998	United States	1212:2126	Lived with smoker; smoke in workplace	Questionnaire	Carotene and HDL cholesterol lower Intake of alcohol and fat higher
Howard et al (20), 1998	Nevada	38:36	Smoke in workplace	Serum micronutrients	α-Tocopherol and β-carotene higher
Osler (21), 1998	Denmark	495:676	Partner smokes	Interview or questionnaire	Men: intake of fruit, whole-meal bread, oatmeal, and cakes lower; intake of meat, ice cream, soda, coffee, and beer higher Women: intake of oatmeal lower; intake of animal fats, white bread, and coffee higher
Tribble et al (22), 1999	United States	44:55	h/wk	Questionnaire + plasma ascorbic acid	Vitamin C intake and plasma ascorbic acid lower
Curtin et al (23), 1999	Switzerland	81 (home):698	≥1 h/wk for ≥1 y; home, work, and leisure	Interview	Intake of fibers, complex carbohydrates, iron, β-carotene, cereals, vegetables, lean meat, and skim milk lower

[1] HK, Honk Kong; S, Sweden; US, United States.

lower plasma ascorbic acid concentrations in passive smokers than in unexposed persons. However, in another study, persons exposed to passive smoking in the workplace had higher serum concentrations of β-carotene and α-tocopherol than did those not exposed to tobacco smoking in the workplace, but only 74 persons were observed (20). The present study was conducted to further address this question by assessing the relation between passive smoking and serum micronutrient concentrations in a larger study population (n = 1590) and for a broader range of micronutrients.

SUBJECTS AND METHODS

Two resources were used to conduct this cross-sectional study: *1*) the Washington County, MD, serum bank (CLUE I) and *2*) a private census conducted in Washington County. The serum bank study was the source of serum micronutrient information and smoking status (whether the individual smoked), and the census was the source of passive smoking data (whether the individual was exposed to environmental tobacco smoke in the household). The serum bank was established in the autumn of 1974 and the census was conducted shortly thereafter, in the summer of 1975. Because of the narrow time interval (7–10 mo) separating the blood draws from the census, these 2 data sources were considered to relate to a single time point for the purposes of this study.

Beginning in the 1980s, the serum bank has been used to conduct many nested case-control studies of micronutrients in relation to cancers. The pool of potential subjects for the present study comprised individuals who donated blood in 1974 and were subsequently selected as control subjects for these nested case-control studies (n = 2142); for the 53 persons whose serum had been assayed twice, data were limited to the initial assay. From this group, 1717 were linked to the private census, the source of passive smoking data, on the basis of name and birth date. Seven institutionalized individuals were excluded, as were 29 with a history of cancer. The study population was further limited to index subjects who were heads of households or spouses of heads of households because relatively few persons (n = 87) were other household members, and these do not share the same likelihood of exposure to passive smoking and shared meals as do spouses. The study population of index subjects was limited to heads of households or spouses of heads of households, but the source of the index subjects' exposure to passive smoking could be any household member. The predominant source of exposure to passive smoking at

home was the spouse; 84% of persons exposed to passive smoking had a spouse who smoked. The micronutrient data were most complete for β-carotene and lycopene; the 4 individuals with missing data for either of these were excluded, resulting in a final study population of 1590 individuals. The data collection protocols for the serum bank and the community-wide census are described briefly below.

The serum bank was established through a community-wide campaign to have as many adults as possible participate in a biomedical research program. Participation included donating a sample of blood and filling out a brief questionnaire. At the time of blood collection, a brief interview was administered to obtain information on demographic characteristics, smoking history, and use of selected medications during the 48 h before blood collection. The serum samples were stored at −70 °C until assayed for micronutrient concentrations for the specific nested case-control studies done between 1986 and 1998.

For the private census conducted during the summer of 1975, a questionnaire was mailed to every residential address in Washington County. Interviewers went door to door to collect completed questionnaires or to help residents complete them. Tobacco use histories were collected for all household members aged ≥16.5 y. A total of 90225 persons, an estimated 90% of the population, were enumerated.

Laboratory assays

Serum micronutrients were not assayed at a single time point specifically for this study but were assayed for several nested case-control studies conducted over a 13-y period (1986–1998). During this period, assays were conducted in 4 different laboratories. Consequently, all results were adjusted for year of assay (to account for storage time) and laboratory (to account for differences between laboratories). Samples were protected from light and analyzed by HPLC for retinol, α-tocopherol, γ-tocopherol, total carotene, α-carotene, β-carotene, cryptoxanthin, lutein and zeaxanthin, and lycopene (24, 25).

Statistical analyses

Assessment of tobacco exposure was limited to cigarette smoking data only for both active and passive smoking because of the small number of persons who smoked other forms of tobacco exclusively. Exposure to passive smoking refers to current exposure in 1975. The reports of active smoking in 1974 (serum bank study) and 1975 (census) agreed for 86.8% of the study population. Changes between former and current smoking status were noted for 8.1% of the study population; the remaining 5.1% had changes reported from former or current smoking to never smoking, which may be because of lack of reliability of responses or because different persons reported the information (self versus other household members). Most of these changes involved smokers who smoked a small number of cigarettes per day.

The PROC GLM procedure of SAS (version 6.12; Statistical Analysis Systems, Cary, NC) was used to estimate mean micronutrient concentrations according to active and passive smoking status, stratified by sex and adjusted for laboratory, year of assay, age, education, and marital status. The distributions of the micronutrients tended to be skewed toward high values so the data were log transformed. We thus present geometric means and results of tests of statistical significance that are based on the log-transformed data.

TABLE 2
Frequency distribution of selected characteristics of the study population by sex, Washington County, MD, 1974

Characteristic	Men (n = 789)	Women (n = 801)
Age (y)		
≤44	106	187
45–54	265	257
55–64	256	233
≥65	162	124
Education (y)		
<12	348	355
12	240	296
>12	201	150
Married[1]		
Yes	708	615
No	80	186
Active smoking		
Never	242	482
Formerly	353	121
Currently	194	198
Laboratory of serum assay (n)		
A	226	251
B	39	87
C	193	130
D	331	333

[1] Information about marital status was missing for one man.

The principal variation in the measured micronutrient concentrations between laboratories was that the results for laboratory B were considerably lower than were those for the other laboratories. To address the concern that the values for this laboratory might have skewed the overall results, analyses excluding the assays performed in laboratory B were conducted; because the study results were not significantly altered by this, the results for the total study population are presented. We previously reported results on decreases in serum concentrations of certain micronutrients with storage time (26). The laboratory and duration of storage variables were highly correlated because of the use of certain laboratories during certain periods, so these variables were combined for adjustment purposes.

Passive smoking exposure was analyzed 3 ways: *1*) as a dichotomous yes or no variable, *2*) by dosage (summed number of packs of cigarettes per day of all smokers in the household other than the index subject), and *3*) categorized according to the source of exposure (spouse or other).

RESULTS

The characteristics of the study population (n = 1590) are summarized in **Table 2**. The average age was 54 y (range: 21–87 y). The study population had equal proportions of men and women and was almost exclusively white (99.1%).

Serum concentrations of β-carotene and lycopene were available for all 1590 individuals in the study population and for the percentage of subjects noted for the remaining micronutrients: retinol (80%), α-tocopherol (72%), γ-tocopherol (52%), total carotenoids (72%), α-carotene (72%), cryptoxanthin (66%), and lutein and zeaxanthin (62%). Serum concentrations of total carotenoids, β-carotene, α-carotene, and cryptoxanthin were significantly lower in current smokers than in never smokers for both men and women (**Table 3**). In men, significantly lower serum

TABLE 3
Adjusted geometric mean serum micronutrient concentrations according to active smoking history, stratified by sex[1]

Micronutrient (μmol/L)	Smoking status		
	Never	Former	Current
Retinol			
Men (n = 596)	2.170 ± 0.036	2.286 ± 0.036[2]	2.185 ± 0.036
Women (n = 671)	1.940 ± 0.036	1.934 ± 0.036	1.973 ± 0.036
α-Tocopherol			
Men (n = 560)	24.78 ± 1.03	25.95 ± 1.03	24.90 ± 1.03
Women (n = 586)	38.32 ± 1.02	40.14 ± 1.04	37.88 ± 1.03
γ-Tocopherol			
Men (n = 366)	5.06 ± 1.06	5.05 ± 1.06	4.91 ± 1.07
Women (n = 456)	8.27 ± 1.04	8.80 ± 1.07	8.10 ± 1.06
Total carotenoids			
Men (n = 557)	1.81 ± 0.019	1.66 ± 0.019[2]	1.47 ± 0.019[2]
Women (n = 584)	2.06 ± 0.019	2.11 ± 0.020	1.83 ± 0.019[2]
α-Carotene			
Men (n = 554)	0.038 ± 0.020	0.033 ± 0.020	0.025 ± 0.020[2]
Women (n = 583)	0.061 ± 0.020	0.058 ± 0.020	0.050 ± 0.020[2]
β-Carotene			
Men (n = 789)	0.195 ± 0.020	0.172 ± 0.020[2]	0.147 ± 0.020[2]
Women (n = 801)	0.282 ± 0.019	0.293 ± 0.020	0.239 ± 0.020[2]
Cryptoxanthin			
Men (n = 524)	0.158 ± 0.020	0.128 ± 0.019[2]	0.096 ± 0.020[2]
Women (n = 525)	0.175 ± 0.019	0.172 ± 0.020	0.121 ± 0.019[2]
Lutein and zeaxanthin			
Men (n = 524)	0.366 ± 0.018	0.339 ± 0.018	0.311 ± 0.018
Women (n = 463)	0.367 ± 0.018	0.356 ± 0.019	0.318 ± 0.018
Lycopene			
Men (n = 789)	0.321 ± 0.020	0.346 ± 0.020	0.306 ± 0.020
Women (n = 801)	0.411 ± 0.019	0.444 ± 0.020	0.390 ± 0.020

[1] Geometric mean ± SEM; values adjusted for year of assay, laboratory, age, education, and marital status.
[2] Significantly different from never smokers, $P < 0.05$.

concentrations were also observed in former smokers than in never smokers for total carotenoids, β-carotene, and cryptoxanthin.

The adjusted mean serum micronutrient concentrations according to active and passive smoking status are summarized in **Table 4**. The most consistent pattern of associations to emerge from the many comparisons was the tendency of never and former smokers who were exposed to passive smoking to have lower serum concentrations of total carotenoids, β-carotene, α-carotene, and cryptoxanthin than did those not exposed to passive smoking at home. Among the never and former smokers, the differences that were significant for these carotenoid measures were limited to men for β-carotene, to women for α-carotene, to men who never smoked for total carotenoids, and to all never smokers plus men who formerly smoked for cryptoxanthin. Even when the differences were not significant, serum concentrations of total carotenoids, α-carotene, β-carotene, and cryptoxanthin were lower in persons exposed to passive smoking than in persons not exposed to passive smoking except for one stratum: serum α-carotene concentrations in formerly smoking men.

For the remaining micronutrients, only 2 other significant differences were observed between persons exposed to passive smoking and those not exposed to passive smoking. Serum retinol concentrations were lower in men who currently smoked and who were exposed to passive smoking than they were in currently smoking men not exposed to passive smoking. Serum γ-tocopherol concentrations were higher in formerly smoking men exposed to passive smoking than in formerly smoking men not exposed to passive smoking (Table 4). There were no significant trends in serum micronutrient concentrations according to the number of cigarettes that household members smoked per day (data not shown).

The study population was composed of index subjects who were heads of households or spouses of heads of households, but the index subject could be exposed to passive smoking by a spouse or by other household members. Most of the exposure to passive smoking at home in this study was from spouses, but 16% of the study population was exposed only by household residents other than a spouse. Ancillary analyses were performed to assess the possible influence on the study results of exposure to passive smoking by a spouse compared with another household member. These analyses were performed for the 2 micronutrients (β-carotene and lycopene) with complete data. These analyses indicated no important differences in the association between exposure to passive smoking and serum micronutrient concentrations according to whether the source of exposure was the spouse or another household member (data not shown).

DISCUSSION

This study was conducted to assess whether exposure to passive smoking was associated with serum micronutrient concentrations. An initial characterization of the associations between active smoking and micronutrients showed that, for both men and women, serum concentrations of total carotenoids, α-carotene, β-carotene, and cryptoxanthin were significantly lower in current smokers than in never smokers. These findings are consis-

TABLE 4
Adjusted geometric mean serum micronutrient concentrations according to active and passive smoking status, stratified by sex[1]

	Passive smoking					
	Never smokers		Former smokers		Current smokers	
Micronutrient (μmol/L)	No	Yes	No	Yes	No	Yes
Retinol						
Men	2.11 ± 0.036	2.18 ± 0.037	2.28 ± 0.036	2.41 ± 0.037	2.22 ± 0.036	1.93 ± 0.037[2]
Women	1.96 ± 0.036	1.91 ± 0.036	1.98 ± 0.036	2.02 ± 0.038	1.90 ± 0.036	1.92 ± 0.037
α-Tocopherol						
Men	24.9 ± 1.05	23.6 ± 1.07	25.6 ± 1.04	25.9 ± 1.06	24.7 ± 1.06	24.4 ± 1.08
Women	38.8 ± 1.02	37.7 ± 1.04	40.0 ± 1.05	39.3 ± 1.08	39.8 ± 24.6	39.6 ± 1.06
γ-Tocopherol						
Men	5.2 ± 1.10	5.0 ± 1.13	4.9 ± 1.08	6.1 ± 1.11[2]	5.0 ± 1.11	4.5 ± 1.14
Women	8.2 ± 1.05	8.5 ± 1.08	8.8 ± 1.11	7.9 ± 1.20	8.2 ± 1.11	8.6 ± 1.12
Total carotenoids						
Men	1.87 ± 0.020	1.60 ± 0.020[2]	1.69 ± 0.020	1.51 ± 0.020	1.54 ± 0.020	1.52 ± 0.020
Women	2.12 ± 0.019	2.06 ± 0.020	1.94 ± 0.020	1.74 ± 0.021	1.67 ± 0.020	1.84 ± 0.020
α-Carotene						
Men	0.039 ± 0.021	0.025 ± 0.022	0.030 ± 0.020	0.031 ± 0.021	0.024 ± 0.022	0.024 ± 0.022
Women	0.066 ± 0.020	0.057 ± 0.020[2]	0.060 ± 0.021	0.039 ± 0.023[2]	0.045 ± 0.021	0.052 ± 0.022
β-Carotene						
Men	0.190 ± 0.021	0.129 ± 0.022[2]	0.199 ± 0.020	0.164 ± 0.021[2]	0.155 ± 0.021	0.164 ± 0.021
Women	0.298 ± 0.020	0.268 ± 0.020	0.302 ± 0.020	0.256 ± 0.022	0.193 ± 0.020	0.222 ± 0.020
Cryptoxanthin						
Men	0.179 ± 0.020	0.116 ± 0.021[2]	0.128 ± 0.020	0.102 ± 0.021[2]	0.098 ± 0.020	0.093 ± 0.021
Women	0.187 ± 0.019	0.153 ± 0.020[2]	0.157 ± 0.020	0.124 ± 0.022	0.125 ± 0.020	0.134 ± 0.021
Lutein and zeaxanthin						
Men	0.375 ± 0.019	0.331 ± 0.019	0.324 ± 0.019	0.338 ± 0.019	0.326 ± 0.019	0.329 ± 0.020
Women	0.381 ± 0.018	0.373 ± 0.019	0.334 ± 0.019	0.348 ± 0.020	0.332 ± 0.019	0.330 ± 0.020
Lycopene						
Men	0.326 ± 0.021	0.368 ± 0.022	0.370 ± 0.020	0.366 ± 0.021	0.291 ± 0.020	0.288 ± 0.021
Women	0.412 ± 0.019	0.441 ± 0.020	0.432 ± 0.020	0.432 ± 0.021	0.391 ± 0.020	0.365 ± 0.021

[1] Geometric mean ± SEM; values adjusted for laboratory, year of assay, age, education, and marital status.
[2] Significantly different from those not exposed to passive smoking, $P < 0.05$.

tent with associations observed previously between active smoking and circulating concentrations of total carotenoids (27–29), β-carotene (30–38), α-carotene (30–32, 36, 37), and cryptoxanthin (31, 32, 36, 37). Retinol (27, 28, 34, 36, 39), the tocopherols (28, 32, 33, 36–38), lutein and zeaxanthin (34, 37), and lycopene (29, 30, 32, 34, 37) were not consistently observed to be associated with active smoking status in previous research, which is also in keeping with the findings of the present study.

With respect to the association between exposure to passive smoking and serum micronutrients, the primary finding of this study was that, in persons who were not current smokers, those who lived with smokers tended to have lower serum total carotenoid, α-carotene, β-carotene, and cryptoxanthin concentrations than did those who lived in households with no smokers. Interestingly, these carotenoid measures are the same ones that were observed to be inversely associated with active smoking both in the present study and in previous research. Nonsmokers who were exposed to passive smoking at home had serum concentrations of these carotenoids that were almost uniformly lower than in persons not exposed to passive smoking at home, but these differences were significant for only about one-half of the comparisons made. Of the significant differences, there was no consistent pattern observed between men and women or between index subjects who were former or never smokers.

The nutrients that were not associated with active smoking—α-tocopherol, γ-tocopherol, lutein and zeaxanthin, and lycopene—also showed little evidence of being associated with exposure to passive smoking. In current smokers, serum retinol concentrations were significantly lower in those exposed to household passive smoking than in those not exposed, but the lack of association observed in never and former smokers casts doubt on the relevance of this observation. This exception aside, in active smokers, exposure to passive smoking was not associated with serum concentrations of any of the micronutrients studied.

The important associations observed in the present study were thus confined to individuals who were not current smokers and to the micronutrients most strongly associated with active smoking. Any additional contribution, beyond that of active smoking, that passive smoking may have on serum micronutrient concentrations may be difficult to detect in smokers because of the overwhelming influence of active smoking.

The specific pattern of associations observed (ie, passive household smoke exposure was associated with lower serum concentrations of total carotenoids, α-carotene, β-carotene, and cryptoxanthin) may reflect genuine associations given that these micronutrients are also associated with active smoking. However, the lack of dose-response trends raises the index of suspicion about this being a chance finding.

The results suggest a few possible hypotheses for future testing. Passive smoke exposure, as a source of oxidative stress (40, 41), could result in lowered circulating micronutrient concentrations by directly depleting antioxidant micronutrients (42, 43). The fact that passive smoking was associated primarily with lower circulating concentrations of the carotenoids that are associated

with active smoking in the present study is compatible with the mechanism of action being an oxidative stress pathway.

Alternatively, because cigarette smokers have poorer diets than do nonsmokers (44), households with a smoker present may have poorer diets than do households with no smokers, resulting in less consumption of fruit and vegetables and hence lowered circulating micronutrient concentrations. Without information concerning dietary intake, we were unable to directly explore this hypothesis. However, the results of previous studies provide some support for this line of reasoning. In one study, wives' smoking habits were significantly associated with their husbands' β-carotene consumption (45). In another study it was observed that, in nonsmoking men, those whose partner smoked had intakes of fruit, boiled vegetables, raw vegetables, and juice that were 9%, 4%, 11%, and 17% lower, respectively, than the intakes of those whose partner was not a smoker (21). These results lend credence to the notion that exposure to passive smoking in the home is associated with lower intake of carotenoids. However, it is not obvious why the associations would be limited to the carotenoids associated with active smoking and not observed uniformly for all the micronutrients studied. One possibility in this regard is that β-carotene is the best biomarker of fruit and vegetable consumption (46); however, this argument does not seem tenable because, although the circulating concentrations of total carotenoids, α-carotene, and cryptoxanthin were all strongly correlated with serum β-carotene concentrations, so were lutein and lycopene concentrations.

A few additional considerations should be borne in mind when the findings of the present study are interpreted. Although we treated this as a cross-sectional study, it was not a cross-sectional study in the traditional sense. The assays of the stored samples were performed over a range of years and in different laboratories. We adjusted statistically for these factors in assessing the associations between passive smoking and serum micronutrients, but even statistical adjustment does not achieve the uniformity in outcome assessment that would have been accomplished if all the assays were performed during the same period by the same laboratory. It is reassuring that the established associations observed between micronutrient concentrations and active smoking were in the expected directions. The measure of passive smoking was specific to the household, but household exposure is the exposure source of primary interest when exploring the dietary link because of the potential importance of shared diets, as discussed above. The measurement of household smoking, as opposed to smoking only by the spouse, is a strength. Our finding that the associations between passive smoking and serum micronutrient concentrations were consistent whether the exposure was from the spouse or from other household members may be helpful in attempts to interpret the results of studies that rely solely on spouse smoking.

In summary, our primary finding was that nonsmokers exposed to passive smoking at home tended to have lower concentrations of some, but not all, carotenoids than did those with no smokers at home. The micronutrient measures that were lower in those exposed to passive smoking were total carotenoids, α-carotene, β-carotene, and cryptoxanthin—the same micronutrients that were lower in active smokers than in nonsmokers. As expected, the differences between those exposed and those not exposed tended to be smaller for passive smoking than for active smoking. The consistency of the associations observed for active and passive smoking, taken together, indicates that passive smoking is associated with lower circulating concentrations of selected carotenoids. If this result is replicated, studies with both serologic and dietary intake data will be required to unravel whether the association is due to a pathway that involves shared diets, to direct depletion of antioxidant nutrients, or to a combination of the 2 pathways.

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